DEVELOPMENT OF RADIOIMMUNOASSAYS FOR MEASUREMENT OF ABSCISIC ACID AND GIBBERELLINS DURING FLORAL INDUCTIVE TREATMENTS IN *Citrus latifolia* Tan.

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

DEVELOPMENT OF RADIOIMMUNOASSAYS FOR MEASUREMENT OF ABSCISIC ACID AND GIBBERELLINS DURING FLORAL INDUCTIVE TREATMENTS IN *Citrus latifolia* Tan.

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The hormonal control of citrus flowering was investigated by utilizing several approaches. Water stress for as little as 2 weeks induced flowering in container grown 'Tahiti' lime (*Citrus latifolia* Tan.) trees. Low temperature (18°C day/10°C night) induced a time dependent flowering response like that of moderate water stress. Low leaf xylem pressure potentials, as compared to controls were found only under water stress treatment, suggesting that a common stress-linked event, separate from low plant water potential, is involved in floral induction. Leafless, immature cuttings were induced to flower by water stress treatment, suggesting that leaves are not essential for a floral inductive response.

Total abscisic acid (ABA) levels in leaves and buds increased as the duration of floral inductive stress increased and were higher under water than low temperature stress conditions as measured by radioimmunoassay (RIA). Total ABA

levels decreased when water stress was alleviated by rewatering. Free ABA in leaves changed in a similar pattern to that noted for total ABA in trees grown under the above stress conditions. Levels of free ABA were less after water or low temperature than before stress and fell sharply after alleviating water stress in trees. Levels of free ABA were 4- to 35-fold lower than total ABA.

Antisera against gibberellins (GA) were produced by immunizing rabbits with a GA_4-adipic acid-bovine serum albumin immunogen. The antisera significantly cross reacted with GA_1, GA_7, and GA_9. Gibberellin levels in leaves and buds collected from trees grown under water stress were dissimilar to those levels measured in low temperature stressed tissues as measured by GA RIA. Gibberellin levels decreased in leaves and increased in buds when water stress was alleviated by rewatering. The increased GA levels in buds corresponded to the alleviation of water stress. Gibberellins and ABA may play a role in water stress-induced bud dormancy and budbreak. Water and low temperature stress treatments induce flowering in 'Tahiti' lime, but the levels of ABA and GA do not change in a similar manner as a result of stress treatment.

CHAPTER I
INTRODUCTION

Flowering in citrus trees is essential for the production of citrus fruit and is an important event for the maintenance of a world-wide commercial horticultural enterprise. Unfortunately, the flowering process of citrus trees is understood to a limited extent. Environmental factors such as temperature and photoperiod as well as cultural practices like gibberellic acid spray have gathered a large share of the flowering research emphasis over the last 30 years in citrus. Many of the citrus flowering studies have been conducted with field grown citrus trees where the floral inductive conditions were poorly controlled or characterized. In addition, more than one floral inductive treatment had not been used in these previous studies to compare plant's physiological and biochemical similarities and differences in the flowering process. The perception and integration of these environmental or cultural factors into internal biochemical control have not been investigated to any significant extent.

In the past, it has been difficult to study many of the internal biochemical controls governing the flowering process. Plant growth regulators (PGRs) which were thought to exert great control over flowering in citrus were inaccesible in the chemically complex tissues of the citrus tree and it was impossible to identify and quantify subtle PGR changes in those tissues where the flowering process may

be subject to environmental control. The recent developments of PGR immunoassays have allowed for the investigation of changing PGR levels in limited quantities of plant tissues with little purification of plant extracts prior to assay. However, these techniques were limited to only a few select laboratories. Our goal was to develop immunoassays in order to quantify those PGRs which were thought to play a controlling role in the flowering process of citrus. The approach taken in the experiments reported here was to develop and characterize several floral inductive treatments in container grown 'Tahiti' lime trees. Container grown trees were used because they allowed for control of floral inductive conditions in the greenhouse and growth chamber. Moreover, plant physiological and biochemical similarities and/or differences among several floral inductive treatments and PGR levels could be correlated and a theory for PGR involvement in the flowering process of citrus could be developed. The success of this approach would be helpful toward gaining a better understanding of citrus flowering.

CHAPTER II
LITERATURE REVIEW

Citrus Flowering

Flowering is the first step toward annual citrus fruit production. The general sequence of events that lead to citrus flowering under subtropical conditions in the northern hemisphere is flower induction, which generally occurs in early winter (December through January); differentiation, which occurs in late January; and the uninterrupted development of floral organs, which leads to the opening of flowers in March through April (1, 5, 72). However, floral induction has recently been reported to occur in Citrus sinensis as early as November (62). Under the climatic conditions of the tropics, some citrus cultivars produce flowers throughout the year (72). Lemon is the most widely known example of this behavior. Flower induction apparently occurs more than once a year. The stimuli regulating this response are not clear; however, water stress is commercially used to induce flowering in lemon (73). There have been a substantial number of studies conducted that relate to flowering of citrus and are reviewed in Volume II of the Citrus Industry (13, 18). The specific control of citrus flowering is not well understood; however, it is generally believed that plant growth regulators (PGRs) are involved. The relevant literature concerning environmental and internal control of flowering in citrus is reviewed here. Moreover, this review will

cover literature that is related to the development of immunological assays that have been used in these studies to help elucidate the role of PGRs in the internal control of citrus flowering.

Environmental Control of Flowering

Environmental control of flowering in citrus is achieved climatically or through the cultural practices of the grower. The most commonly believed environmental controller of flowering in citrus is thought to be exposure to periods of reduced temperature during the winter months (38, 59, 72, 74, 78, 79). These findings have resulted from both greenhouse and growth chamber experiments as well as from observations of trees growing in the field. The temperatures required to induce flowering range from 15 to 20°C (day) and 5 to 15°C (night) (38, 59, 77), but the critical amount of time needed to induce flowering under low temperature conditions has not been reported. It was reported that air temperature was more important for regulation of flowering than root temperature in Citrus sinensis (38, 79). Attempts were made to induce flowering in Citrus sinensis by utilizing low root temperatures (11 and 15°C) together with non-inducive air temperatures (22 and 27°C). Flowering could not be induced by low root temperature and the lack of flowering was not correlated with a general inhibition of growth, which had been due to low root temperature (79). High temperature above 30°C inhibited flower formation under the growing conditions of the growth chamber (77). There is good evidence in Citrus

that flowering is day neutral (77) and that changing photoperiod does not affect flowering. Flowering has been shown to be controlled by photoperiod only under moderate temperature (24°C day/19°C night) conditions (59).

There are several practices which growers can use to regulate flowering in citrus. Certain lemon (Citrus limon (L.) Burm.f.) cultivars were induced to flower because of an extended period of water stress (11, 82). Although it is well known that general water stress in citrus promotes flowering, there are only 2 reports in the literature addressing the topic. These reports do not determine whether water stress is truly a floral inductive treatment. Moreover, the research has been conducted in the field with mature trees where little control or characterization of water stress could be achieved. Practices such as autumn pruning (6), excessive nitrogen fertilization (56), application of gibberellic acid (17, 35, 36, 37, 69, 72, 73, 75), and excessive or late cropping (42, 43, 48, 49, 50, 61, 71) can reduce flower bud formation and often the subsequent yield in the following season. Although a reasonable amount of research data have been generated with regard to the environmental control of flowering in citrus, the amount of research data concerning the endogenous, internal chemical control of flowering is sparse.

Internal Control of Flowering

The exogenous application of gibberellic acid (GA) and the inhibition of citrus flowering have led to the speculation that GA controls flowering. Although it is true that

GA application can inhibit the formation of flowers in citrus, there has been only one report of the measurement of GA in citrus as it relates to flowering (35). In that report a negative correlation was reported between length of flowering branches and their flower load. Vegetative branches are the longest and the levels of native GA-like substances were highest in the vegetative flowerless branches (35). As the number of leaves decreased and the number of flowers increased (from vegetative to generative shoots), the levels of GA-like substances also decreased. Other circumstantial evidence implicates GA in control of citrus flowering. The use of GA synthesis inhibitors such as (2-chloroethyl)trimethylammonium chloride (chlormequat), or succinic acid-2,2-dimethylhydrazide (daminozide) can induce larger flower numbers under certain conditions (74, 75). This fact lends more credence to the theory of GA control of flower formation.

The way in which GA may control flowering at the present time is unknown and little research has been done in citrus to clarify the problem. However, one report showed that synthesis and total protein levels decreased in buds of 'Shamouti' orange trees during the flower formation period. The application of GA during the same period resulted in additional protein bands as compared to untreated controls in polyacrylamide gel electrophoresis (76). It was speculated that these additional bands may be proteins that inhibited flowering.

The levels of carbohydrates are also thought by some to be internal controllers of flowering in citrus (33, 42, 43, 48, 49, 50). The levels of carbohydrates in leaves were found to decrease as fruit were left on trees later into the season. The decrease in total carbohydrate levels correlated with a reduction in flower formation, fruit set, and yield the following year (42, 43 48, 50). Carbohydrate levels were found to be associated with alternate bearing of 'Wilking' mandarin trees. Apparently, the levels of carbohydrates increased due to fruit thinning along with the rate of flower bud differentiation (33). The correlation was best between fruiting and starch levels and poor with sugar or total carbohydrate levels. Starch was found to accumulate in the roots of 'Wilking' mandarin in the "off" year of an alternately bearing tree. However, Lewis et al. (61) rejected the hypothesis that carbohydrate levels are responsible for the lack of flowering in 'Wilking' mandarin during the "off" years because they found that thinning changed the production cycle without significantly affecting carbohydrate levels. Similarly, Jones et al. (49) found that the effectiveness of thinning treatments was not clearly correlated with carbohydrate levels in 'Valencia' orange. There is disagreement in the conclusions drawn from these studies and the discrepancies may have resulted from using different plant tissues as well as differing techniques to measure carbohydrates.

It is well documented that carbohydrate levels do change in leaves, roots, buds, etc. as a result of crop load

or during the floral inductive period in citrus; however, it is unlikely that a carbohydrate can directly control a complex process such as flowering. On the other hand, gibberellic acid has been shown to inhibit flower formation in citrus. It has been difficult to measure gibberellin levels in the small amounts of tissue required for the analysis of PGRs in citrus buds. The development of immunoassays to quantify PGRs has the potential of overcoming many of the problems associated with the measurement of PGRs and the literature concerning the development of PGR immunoassays is outlined below.

Plant Growth Regulator Immunoassays

The immunoassay was first developed by Berson and Yalow (8) for the quantification of insulin in human plasma and was soon recognized as a powerful tool in endocrinology, clinical medicine and pharmacology. The technique allowed for the quantification of exceedingly low levels of low and high molecular weight compounds, after little sample preparation, in small amounts of tissue in a short period of time (8, 117, 118). Fuchs and coworkers (25, 26, 27, 28) were the first to attempt immunological procedures for the detection and quantification of PGRs. Since these early studies, the scope and usefulness of PGR immunoassays have increased and radioimmunoassays (RIAs) and enzyme-linked immunoassays (EIA) to quantify levels of gibberellins (GA), abscisic acid (ABA), cytokinins, and auxins (14, 108, 109, 110) have been developed.

Principles of the Immunoassay

The immunoassay is based upon the competition of a known amount of labeled antigen and an unknown amount of sample antigen for a finite number of high-affinity binding sites (118). In order to develop a successful immunoassay there must be a suitable availability of specific antiserum, labeled antigen which exhibits a comparable affinity for the antibody as the unlabeled antigen, and a convenient and reliable procedure for separation of antibody-bound from free antigen without interference of antibody equilibrium (108, 109, 110).

Plant growth regulators are low molecular weight compounds. Thus, PGRs must be chemically coupled to higher molecular weight compounds such as albumins, keyhole limpet haemocyananin, or thyroglobulins (proteins) to create PGR-immunogens (19). The synthesis of the PGR-immunogens and the choice of functional groups used in the synthetic procedure depends upon the degree of antibody selectivity required. A high degree of selectivity is obtained when the PGR is most exposed after coupling to the larger molecular weight compound (19). Examples of greater selectivity due to the synthetic procedure used to create the PGR-immunogen include the ABA carbon 4-coupled immunogens. These immunogens have been created by inserting a chemical spacer between the carbon 4 of ABA and the protein carrier bovine serum albumin (104). This coupling procedure allowed antisera to cross react only with free ABA rather than both free and bound ABA which results from the coupling of ABA at

carbon 1 to albumin (101, 103). Another example of antisera selectivity has been noted where immunogen synthesis via the ribosyl moiety of cytokinin ribosides yields antisera reactive with the free bases, 9-ribosides, 9-ribotides, and 9-glucosides, but which discriminate trans zeatin from cis zeatin or dihydrozeatin (63, 105). Antisera with different selectivities can be achieved by varying immunogen synthesis. Each PGR has different functional groups through which conjugation to large carrier molecules can occur. Moreover, other techniques are available for introduction of reactive functional groups to PGRs which lack them (19).

Once a suitable polyclonal or monoclonal antibody has been obtained, it is important to select a suitable tracer antigen. Radionuclides such as ^{3}H (108, 109, 110) or ^{125}I 106, 113) have been used previously for RIA. In the case of ELISA the use of enzymes, notably alkaline phosphatase, have been used to label PGRs (4, 14, 15, 107, 111, 112). After the selection of the tracer has been made, it is important to select the optimum assay conditions and characterize the antibody and a number of reviews deal with these subjects (55, 108, 109, 110). In some cases it has been necessary to chemically modify the antigen prior to analysis in order to maximize the sensitivity of the assay. When carboxyl groups of the antigen have been used to synthesize PGR-immunogens (e.g. auxin (IAA), GAs) it was necessary to methylate antigens in order to restore immunoreactivity (3, 4, 111, 113). The reason methylation is necessary is not completely clear; however, it has been speculated that the antigen

coupling site of the immunogen and the antigen-protein link may constitute part of the overall binding area of the antibodies (110) rather than only the antigen which does not completely occupy the antibody binding site.

Gibberellin Immunoassay

The development of immunoassays for the quantification of GAs in plant tissue extracts has been limited. Both an RIA and ELISA have been developed for GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} (3, 4, 112). These assays were reported to be sensitive. The RIA could measure pmol amounts of GA_3 in unpurified plant extracts and the ELISA could measure fmol amounts of either GA_3, GA_4, and GA_7 methyl ester. Approximately 1 mg of plant tissue was required to measure GA by the immunoassays. Immunogen synthesis was carried out through the carbon 6, COOH functional group of GA, and the immunogen resulted in the production of antisera that were selective for specific GAs. Antisera against apolar GAs proved to be the most selective (3, 4, 113). Only a limited number of GAs have been used to produce GA-directed antibodies. Future approaches to GA isolation and quantification by immunoassay could utilize group-selective assays (109, 110) in combination with a suitable separation system such as high pressure liquid chromatography.

Abscisic Acid Immunoassay

There have been several RIAs and ELISAs developed for ABA (14, 15, 60, 67, 86, 101, 103, 104, 107, 112). In the early assays developed against ABA, the immunogen was

prepared by coupling (±) ABA via carbon 1 to protein carriers. This immunogen resulted in the production of antisera that preferentially cross reacted with (-) ABA rather than (+) ABA, which is the enantiomer found in plants. This result rendered the assay imprecise. This problem was overcome by using (+) ABA immunogens (104). However, the C 1-coupled (+) ABA antisera detected both ABA and ABA-conjugates (101, 103). In order to produce antisera which only reacted with free ABA, the C 4 of ABA was coupled to tyrosylhydrazone and the resulting compound was coupled to albumin to form an immunogen. The antisera obtained selectively recognized free ABA and not ABA-conjugates (104). The same approach was used in the production of ABA monoclonal antibodies and allowed for the detection of less than 5 pg 2-*cis*(+) ABA (67). The antisera obtained against ABA or ABA and its conjugates does not cross react with phaseic acid, dihydrophaseic acid, xanthoxin, or other compounds known to occur in plants and which have similar chemical structure (67, 101, 103, 104).

Auxin and Cytokinin Immunoassay

Radioimmunoassays and ELISAs have been developed for indoleacetic acid (IAA) (84, 85, 106, 111). The assays were sensitive for IAA in the fmol range when IAA as a methyl ester was used as a standard (111). The production of IAA immunogens by the Mannich reaction (85) resulted in the production of antisera that was selective for IAA, but low serum titers were obtained. Antisera with higher titers were obtained by coupling the IAA carboxyl to the protein

carrier. However, the antisera had less selectivity and cross reacted with side chains similar to that of IAA, such as indole-3-acetaldehyde (106, 111). Because of these problems, plant extracts have required some prepurification prior to quantification by IAA immunoassay (110).

The production of cytokinin immunogens is based on the procedure of Erlanger and Beiser (20) where periodate-oxidized ribosides are reacted with amino groups of the carrier protein followed by borohydride reduction (20, 63, 98, 104). Using this type of immunogen, antisera have been obtained that are capable of determining free cytokinin as well as 9-substituted cytokinins (98, 105). Antisera have also been produced that discriminate between trans-zeatin type and isopentenyladenosine type cytokinins (98, 105). These RIAs made use of ^{3}H or ^{125}I for tracers and were sensitive for cytokinin derivatives in the fmol range. By using alkaline phosphatase-labeled isopentenyladenosine and trans-zeatin type cytokinins, an increase of 5- to 10-fold in sensitivity could be achieved (110).

The development of immunoassays for PGRs has allowed for sensitive and specific measurement of PGRs in small amounts of plant tissue samples. In the future it is apparent that many more assays for the quantification of specific compounds in plants will become available. Moreover, it will be possible to use group selective antisera together with high pressure liquid chromatography or other chemical analytical separation techniques to quantify closely related compounds such as cytokinins and gibberellins.

CHAPTER III
CHARACTERIZATION OF WATER STRESS AND LOW TEMPERATURE EFFECTS ON FLOWER INDUCTION IN CITRUS

Introduction

'Tahiti' lime, Citrus latifolia Tan., is a sterile triploid that is vegetatively propagated thereby avoiding a juvenile period that is common to many tree species. Flowers are produced and fruit set and mature on leafy rooted cuttings as well as on plants propagated by air-layering. This unusual habit makes 'Tahiti' lime suitable as a test plant to study flowering in trees (16, 17, 92). Flowering in citrus can be induced by low temperature (16, 20, 59, 77, 78, 79, 92) or water stress (11, 66, 82, 92) and inhibited by applied gibberellin (17, 35, 46, 70, 73, 82). Regulation of flowering by water stress is not common in trees and generally is reported to be effective in tropical and subtropical species (2, 7, 11, 82). These studies, however, have been conducted under varying field conditions and are not descriptive (7) in the sense that the studies do not define the quantitative relationship between imposed stress and the flowering response. These same shortcomings are true for studies involving low temperature regulation of flower induction.

In our effort to understand the chemical control of flowering in 'Tahiti' lime, we felt it important to manipulate flowering in small container grown trees and to have more than one method of flower induction available to

us. By utilizing several floral-inductive treatments, we hope to ascertain whether a common regulatory event or signal controls flower induction. In this chapter we describe the quantitative relationship between low temperature and water stress on floral induction.

Materials and Methods

Plant Material. In most experiments we used 1- to 2-year-old 'Tahiti' lime trees propagated by air-layering or by bud-grafting on to Citrus macrophylla Wester rootstock. The trees ranged in height from 0.5 to 1.0 m and were grown in the greenhouse under South Florida growing conditions (80) in 16-cm black plastic pots in a mix of 1 peat:1 perlite:1 sand, and fertilized regularly with a 20:20:20 NPK soluble fertilizer plus micronutrients. In other experiments, cuttings were obtained from 18-year-old 'Tahiti' lime trees on rough lemon (Citrus jambhiri Lush.) rootstock in Rockdale limestone soil at the Homestead, Tropical Research and Education Center. Cuttings were selected by clipping immature current season's growth, which bore mostly fully expanded, but non-hardened, immature leaves, and were reclipped near the base under water. Leaves were removed so that 5 to 8 nodes and 2 to 4 or no leaves remained, depending upon treatment. Cuttings with one end were stuck in vermiculite and were placed either in the greenhouse or on another bench under intermittent misting.

Water Stress Treatments. Water stress of trees was either continuous or cyclical. Continuous water stress was established by sealing the pot, soil and roots in a plastic

bag with only stem and leaves exposed to the environment and withholding water. Transpirational water loss from each container grown tree was determined by monitoring the daily weight decrease. Lime trees were transpiring approximately 140 ml water/day. From these measurements 67 percent of the amount of water lost per day was added back to the tree daily so that stress could be gradually imposed and leaf drop minimized. When all leaves became wilted and the mature leaves had a xylem pressure potential of at least -3.5 MPa (severe stress as defined by Syvertsen, 95), 100 ml of water per day was added to each in order to approximately replace transpirational water lost and to keep these trees under constant stress. Addition of 100 ml of water per day to soil briefly saturated the soil until it drained to container field capacity. In absolute terms, continuous water stress may not precisely define this sequence of events, but continuous water stress best describes our observations with regard to the water status of these trees. Cyclical water stress was achieved by stressing each tree to the point of wilting as above and then refilling the container to the full capacity, which set the soil at field capacity. The dry (wilting), wet (container soil at field capacity) cycle or continual stress was continued for the duration of each experiment. Control and treatment trees were preconditioned for at least 1 month through maintenance of container soil at field capacity by applying water twice daily through automatic drip irrigation. Leaf xylem pressure potentials were measured predawn and midday by the

pressure bomb technique (89). Leaves were removed at the petiole-blade abscission zone of each leaf, and measurements were made within 30 s of leaf removal. Leaf xylem pressure potential measurements were made at weekly intervals in the time course experiments, and 2 leaves per tree (10 leaves/treatment) were measured at predawn and midday. Only 2 leaves were used from each tree in order to maximize the number of leaves remaining on stressed trees and maintain uniformity of treatment.

Low Temperature Treatments. Growth chamber experiments were conducted at $18^{o}C/10^{o}C$ (day/night) temperatures with 12-h photoperiods at a photon flux ranging from 350-850 $uE/m^2/s$. Prior to placing each tree in the growth chamber, approximately one-half of all branch apicies including 2 to 3 leaves and nodes were clipped off (16). Controls were treated likewise and were grown in the greenhouse under South Florida conditions (80) and $29^{o}C/24^{o}C$ (day/night) temperatures. In these experiments, total shoots produced represent the sum of vegetative, mixed, and generative shoots. Those 3 shoot types are defined here as they have been previously (35, 70, 77). Briefly, vegetative shoots carry leaves only, mixed shoots carry both leaves and flowers, and generative shoots carry flowers only. Tables have been obtained from at least 2 replicate experiments in all cases.

Results and Discussion

Continuous or cyclical water stress for 4 to 5 weeks (from the initiation of reduced water application to

restoration of daily irrigation to container soil field capacity) resulted in flower induction of 'Tahiti' lime (Table III.1). Continuous and cyclical water stress resulted in more total shoot production as well as a significantly greater number of flowers than controls. After these trees had completed flowering, the flowers and fruitlets were removed. The trees were allowed to resume vegetative growth for a period of 2 months under controlled greenhouse growing conditions. The same trees were induced to flower a second time by the above procedure. The rationale for using the same trees in experiment 2 was that flower reinduction in the same population of trees by the same treatment should indicate that our treatments were truly effective since heavy flowering in subsequent flushes does not occur in greenhouse-grown citrus. The results of the second experiment were similar to those of the first. Continuous and cyclical water stress resulted in trees producing more total shoots and flowers than controls, which produced only random and insignificant numbers of flowering shoots and flowers. There were more flowers/plant produced in continuous than cyclical stress of experiment 1, but not in experiment 2. No significant differences were found between continuous or cyclical stress with regard to shoots per plant, shoot type, or flowers per plant, but continuous stress generally resulted in greater numbers of total shoots per plant and flowers per plant. In fact, as long as the severity of stress resulted in prolonged wilting or a leaf xylem pressure potential of -3.5 MPa for 4 to 5 weeks, the

Table III.1 Effect of continuous or cyclical water stress on flower induction in containerized 'Tahiti' lime trees.

Water Stress Treatment	Shoots/ Plant	Shoot Type (%) Vegetative	Mixed	Generative	Flowers/Plant
EXPERIMENT 1[z]					
Control	5.7[y]±0.5	13.0	0	87.0	5.0± 1.4
Continuous	41.7±8.0	23.3	17.4	59.3	145.7±48.5
Cyclical	29.5±4.4	44.1	16.1	39.8	44.2±14.3
EXPERIMENT 2 (Repeat)					
Control	0.3±0.5	0	0	100.0	0.3± 0.5
Continuous	37.7±5.0	11.3	53.6	35.1	75.7±18.1
Cyclical	29.0±5.0	19.0	48.3	32.7	78.5±51.9

[z]Experiment #1 - 2/2/84 to 3/17/84.
Experiment #2 - 5/11/84 to 6/12/84.

These data represent 1 of 2 replicate experiments.
In this experiment, the same 4 tree replicates/treatment were used.

[y]Values represent means ± standard deviations.

flower inductive response was similar and significantly different from controls. Therefore, the continuous stress condition was used because it was easy and allowed for uniformity of treatment.

The above experiment, which had been performed at two different times of the year including that when 'Tahiti' lime typically does not flower, indicated that flowering could be induced in containerized lime trees by a period of water stress lasting for a 4 to 5 week period. In order to more clearly define the duration and severity of water stress needed to induce flowering, leaf xylem pressure potentials were measured at weekly intervals over a 4 to 5 week period in a population of trees that were stressed. The level of moderate stress maintained above controls at predawn and midday for each time interval measured (Table III.2). We defined moderate levels of stress (-2.1 to -3.0 MPa) as those levels intermediately between control and severe stress (-3.5 MPa) (95). Midday leaf xylem pressure potentials were generally lower and measurements less variable than those at predawn, except at week 5 where predawn stress was as great as that at midday. Intertree variability and daily climatic changes were presumably responsible for the variability in the pressure potential measurements. Control trees produced the least number of shoots/plant and those shoots were vegetative. More shoots/plant were produced as a result of water stress and the numbers generally increased in trees exposed to greater durations of water stress. Flowering was induced after 2

Table III.2 Effect of moderate water stress over time on leaf xylem pressure potential and flower induction in 'Tahiti' lime.

Duration of Water Stress (wks)	Leaf Xylem Pressure Potential (MPa) Predawn	Midday	Shoots/Plant
Control	-0.34±0.08[y]	-1.48±0.15	4.50[x]±1.9
2	-0.90±0.42	-2.25±0.08	6.25±2.2
3	-1.62±0.82	-2.21±0.25	8.00±2.6
4	-0.87±0.09	-2.89±0.23	9.75±3.0
5	-2.89±0.62	-2.83±0.19	9.75±1.5

[z] % flowering shoots = sum of mixed and generative shoot percentages.

[y] Values represent the means of 10 leaf replicates/treatment ± standard deviations.

[x] Values represent the means of 5 tree replicates/treatment ± standard deviations.

Table III.2 -- Extended

Shoot Type (%)			Flowers/Plant	(%)[z] Flowering Shoots
Vegetative	Mixed	Generative		
100.0	0	0	0	0
68.0	16.0	16.0	3.0±0.82	32.0
46.9	21.9	31.2	5.0±2.16	53.1
43.6	20.5	35.9	9.0±2.16	56.4
10.3	56.4	33.3	21.0±8.04	89.7

weeks of stress. The percent flowering shoots and number of flowers per plant increased with time under stress. The highest percentage of flowering shoots and flowers per plant were found after 5 weeks of water stress. Apparently, moderate levels of stress can induce flowering in a relatively short period of time (2 weeks), but the inductive response is much greater after an extended time period (5 weeks).

In a similar experiment, 'Tahiti' lime trees were severely stressed as indicated by leaf xylem pressure potentials ranging from -3.25 to 3.67 MPa (Table III.3). Predawn and midday leaf xylem pressure potentials were significantly different from controls at each measured time. Predawn and midday stress measurements were significantly different from one another at 2 weeks, but thereafter, pressure potentials were not different from one another, and a constant level of water stress prevailed in these trees throughout the experiment. At these severe stress levels, a less variable leaf xylem pressure potential was maintained than those measured for moderate stress (Table III.2) indicating that control of stress (water potential) was obtained under severe water stress conditions. As in the preceding experiment, control trees produced very few shoots and those shoots produced were vegetative. On the other hand, severe water stress when compared to moderate water stress resulted in much greater numbers of shoots and flowers per plant as well as increased percentages of flowering shoots at all measured time intervals. Flowering

Table III.3 Effect of severe water stress over time on leaf xylem pressure potential and flower induction in 'Tahiti' lime.

Duration of Water Stress (wks)	Leaf Xylem Pressure Potential (MPa) Predawn	Midday	Shoots/Plant
Control	-0.24±0.05[y]	-1.38±0.29	3.25[y]±2.0
2	-2.00±0.35	-3.25±0.07	70.50±18.0
3	-3.41±0.83	-3.67±0.24	45.00±12.0
4	-3.56±0.25	-3.66±0.21	49.00±25.0
5	-3.54±0.27	-3.58±0.23	49.80±10.2

[x] % flowering shoots = sum of mixed and generative shoot percentages.

[y] Values represent the means of 10 leaf replicates/treatment ± standard deviations.

[z] Values represent the means of 5 tree replicates/treatment ± standard deviations.

Table III.3 -- Extended

Shoot Type (%)			Flowers/Plants	(%)[z] Flowering Shoots
Vegetative	Mixed	Generative		
100.0	0	0	0	0
16.0	23.7	60.3	246.75±15.8	84.0
8.9	36.7	54.4	97.2±33.2	91.1
6.9	26.6	66.5	144.6±41.6	93.1
6.4	34.1	59.4	168.9±22.8	93.5

trends between moderate and severely water-stressed trees were dissimilar over time, with severely water stressed trees producing the same number of flowers and flowering shoots at each measured time interval. Under moderately water stressed conditions, water stress duration was a factor regulating the flower inductive response. The flowering response appears to be time dependent when regulated by moderate levels of water stress, but at some point floral induction is more immediately reached under conditions of more severe water stress.

Low temperature stress 18°C/10°C (day/night) time course experiments were conducted with containerized 'Tahiti' lime trees growing in the growth chamber as previously reported (10, 59, 77). Leaf xylem pressure potentials did not significantly differ from one another at predawn except at the 4-week time interval (Table III.4). At midday, however, control trees growing in the greenhouse had significantly more negative pressure potentials at all intervals measured. Control trees, as in the previous water stress experiments, produced very few shoots per plant, and those produced were vegetative (Tables III.3 and III.5). Flowering was induced in trees after having been in the growth chamber for as little as 2 weeks, but the response was not as great as that of severely water stressed trees within the same time period (Table III.5). The low temperature stress of the growth chamber resulted in a flower inductive response like that of moderate water stress, but apparently not through a common reduction in leaf xylem

Table III.4 Effect of low temperature over time on leaf xylem pressure potential in 'Tahiti' lime.[z]

Duration of Continual Low Temperature Stress (wks)								
0	2	C[y]	4	C	6	C	8	C
Leaf Xylem Pressure Potential (MPa)								
Measured at predawn:								
-0.360	-0.400	-0.166	-0.479	-0.373	-0.413	-0.426	-0.340	-0.326
±0.098[x]	0.105	0.041	0.055	0.101	0.069	0.043	0.092	0.064
Measured at midday:								
-1.446	-0.613	-1.306	-0.623	-1.926	-0.633	-1.840	-0.500	-1.94
±0.203	0.109	0.118	0.072	0.086	0.052	0.149	0.066	0.08

[z] Low temperature conditions utilized in these experiments were 12 hours each 18°C day/10° night.

[y] C = control treatments at each time measurement.

[x] Values represent means of 10 leaf replicates/treatment ± standard deviation.

Table III.5 Effect of low temperature over time on flower induction in 'Tahiti' lime.[z]

Duration of Reduced Temperature (wks)	Shoots[y] /Plant	Shoot Type (%)			Flowers/Plant	Flowering[x] Shoots (%)
		Vegetative	Mixed	Generative		
Control	5.20±0.4	100.00	0	0	0	0
2	6.20±3.8	54.84	12.90	32.26	5.60±4.03[z]	45.16
4	9.80±3.8	44.90	18.37	36.73	14.20±6.76	55.10
6	13.20±3.7	33.33	36.37	30.30	25.40±10.02	66.67
8	15.20±5.8	22.37	14.47	63.16	30.00±9.97	77.63

[z] Low temperature conditions utilized in these experiments were 12 hours each 18°C day/10°C night.

[y] Values represent means from 5 tree replicates/treatment ± standard deviations.

[x] % flowering shoots = sum of mixed and generative shoot percentages.

pressure potential. A different signal, mediated through a common mechanism, may be regulating floral induction. Severe water stress rather than low temperature stress consistently produced the greatest number of flowers and flowering shoots. Floral induction best describes the floral response observed after both water and low temperature stress. This belief is based upon results obtained from other experiments which will be presented elsewhere (S. M. Southwick and T. L. Davenport, submitted for publication J. Amer. Soc. Hort. Sci.) indicating that lime trees forced to produce shoots by branch pruning produced a greater percentage of flowering shoots after imposing the above stress treatments.

A final experiment was conducted to determine if water stress would induce flowering on cuttings that had been obtained from trees growing in the field. Cuttings (see Materials and Methods) were separated into 2 populations: those with leaves and those with leaves removed. From each population, 1 set was placed in the greenhouse and allowed to dehydrate by only occasionally irrigating (water stress), and another set was put on a different bench under intermittent mist (non-stressed) to alleviate dehydration (40). During the period in the greenhouse prior to flower production, all leaves, except for the most immature, wilted and abscised. After 33 days had elapsed, those cuttings that had been placed in the greenhouse (desicated), both leafy and those with leaves removed, produced flowers (Table III.6). The greatest number of shoots, flowers, and

Table III.6 Effect of leaves and misting on flowering of immature 'Tahiti' lime cuttings.

Treatment	Total Shoots (no.)	Shoot type (mean) Vegetative	Mixed	Generative	Flowers (no.)	Flowering Shoots (%)
Greenhouse						
No Leaves	8.3[z] ± 1.5	5.6 ± 2.1	0	2.7 ± 0.6	3.0 ± 1.0	32.0
Leaves	16.7 ± 1.1	5.0 ± 3.6	2.7 ± 1.1	9.0 ± 1.7	22.7 ± 11.7	70.0
Mist Bed						
No Leaves	-	-	-	-	-	-
Leaves	14.7[y] ± 6.5	8.0 ± 4.0	2.3 ± 1.5	4.3 ± 2.1	10.3 ± 1.5	45.4

[z] Each value represents the mean of 3 experiments where at least 10 replicates/treatment were used. ± = standard deviation.

[y] Cuttings in the mist bed did not have shoots or flowers after 33 days, therefore, those cuttings were removed from the mist bed, placed on another bench in the greenhouse and data recorded 5 weeks later.

flowering shoots were produced on cuttings that had initially borne leaves. Nevertheless, those cuttings that had their leaves manually removed produced both vegetative and flowering shoots. Since immature, leafy cuttings were selected for these experiments, it is improbable that a previously stored floral message was present. Furthermore, flowers produced on leafless cuttings suggest that it is not essential for leaves to be present for floral induction and that perception of flowering cues occurs within the shoot, or as most likely in the bud itself. Although it has been speculated that citrus roots may produce a substance(s) which can be transported to shoots and exert control over bud-break and flowering (38). These immature cuttings used in the experiments reported here, never flower in the field while attached to the tree until they go through a period of maturation.

Cuttings that were placed in the mist bed did not produce shoots or flowers. Therefore, after the same 33 day period, these cuttings were removed from the mist bed and placed on an open bench in the greenhouse, and after another 5 weeks those cuttings bearing leaves produced vegetative and flowering shoots as a result of water stress (Table III.6). Cuttings without leaves which had been placed in the mist bed did not produce any new shoots and eventually died.

In conclusion, 'Tahiti' lime trees preconditioned at container soil field capacity for about 1 month can be severely water stressed for a period of as little as 2 weeks

and consistent flower inductive responses obtained. The floral response seems to be time dependent under conditions of moderate water stress and low temperature. However, floral induction from low temperature when compared to water stress is not mediated through a common decrease in leaf xylem pressure potential. Immature leafless cuttings can produce flowering shoots under water-stress conditions, indicating that leaves are not essential for flower induction in ´Tahiti´ lime.

CHAPTER IV
INVESTIGATIONS OF HORMONAL CONTROL OF CITRUS FLOWERING: DEVELOPMENT OF A RADIOIMMUNOASSAY FOR THE MEASUREMENT OF ABSCISIC ACID IN LEAVES AND BUDS OF 'TAHITI' LIME

Introduction

Abscisic acid (ABA) is a plant growth regulator which is typically associated with general stress phenomena in plants (65, 68, 100). The levels of ABA and ABA-conjugates (9, 53, 81) increase in plant tissues during temperature (100) and especially water stress (9, 34, 68, 100, 119, 120, 122) and a physiological role for ABA has been suggested most often for stomatal control (65, 68, 100). It is thought that free ABA produced in guard cells is responsible for changing K+ levels, which changes cellular water potential and alters cell turgor (65, 100, 112). The regulation of stomatal physiology by ABA is thought to be one way in which plants regulate water loss in order to adapt to various environmental stresses (65, 68, 100).

Abscisic acid and ABA-conjugates have been found to increase during citrus fruit maturation and have been related to exocarp senescence and the transition of chloroplasts to chromoplasts in this tissue (10, 31, 34). The accumulation of ABA or possibly the 2, trans-isomer of ABA in fruit has been purported to influence bud dormancy and contribute to biennial-bearing in 'Wilking' mandarin (30, 47). The specific identities of ABA-conjugates, however, have not been described.

A speculative role for ABA flowering of selected short day plants has been suggested (121). Even though an ABA application alone cannot induce flowering under absolutely non-inductive conditions, short day plants such as Pharbitis (39), Lemna (41), and Chenopodium (54, 91) can be promoted to flower by ABA application if test plants had been slightly induced. Application of ABA (100 ng/ml) to the culture medium stimulated flower formation in vegetative stem explants of Torenia which otherwise had little flower forming capacity (96). There are no reports, however, of any link between ABA levels and flowering in citrus or in any tree species for that matter.

Until recently, it has been difficult to measure ABA in small amounts of plant tissue and correlate a role for ABA with the flowering process of citrus. Advances in immunological methods (101, 103, 104, 107, 108, 109, 110, 112, 114) have minimized the need for plant tissue extract purification and allow for analysis of many samples using a minimal amount of tissue. In these studies reported here we have developed a radioimmunoassay for measurement of total ABA as described previously (60, 86, 103, 104). 'Tahiti' lime trees were grown under the floral inductive conditions of severe water and low temperature stress (Chapter III). Since low temperature and water stress lead to the accumulation of ABA, we wondered whether the accumulation could be linked to control of flowering in lime. The changes in ABA levels in leaf and bud tissues were measured

by radioimmunoassay and compared with floral inductive treatment.

Materials and Methods

Plant Material and Extraction Procedure. In these experiments, 1- to 2-year-old container grown 'Tahiti' lime (Citrus latifolia tan.) trees were grown in the greenhouse or growth chamber as described in Chapter III. Five tree replicates/treatment were severely water stressed (at least -3.5 MPa leaf xylem pressure potential, Chapter III) and leaf and bud tissue were collected from trees after 2, 3, and 4 weeks of continual water stress. Water stress was alleviated by rewatering the container soil to field capacity and leaf and bud tissue were collected 1 and 10 days later. In addition, 5 tree replicates/treatment were placed in the growth chamber under low temperature conditions as described previously (Chapter III). Leaf and bud tissue were collected after 2, 4, and 6 weeks of growth in the chamber. Immediately after each water or low temperature stress interval had elapsed, leaves or buds from each tree replicate were removed and washed with distilled water, towel dried, pooled together, frozen in liquid N_2 and ground in a mortar with a pestle into a fine powder. The tissue was stored in the freezer at -18°C for less than 90 days until use. The extraction procedure of Weiler (103) was followed with slight modification. Fifty to 100 mg of frozen powdered tissue was extracted with 90% methanol (MeOH) containing 10 mg/l 2, 6-di-t-butyl-4-methyl phenol (BHT) (69) (200 ul/mg frozen tissue) for 2 days in the dark

at 4°C. The tissue was shaken at regular intervals daily and the supernatant collected after centrifugation at 7,000 rpm for 10 minutes. Aliquots of the extract were diluted 1:10 or 1:25 with phosphate buffered saline (PBS, 0.01 M Na phosphate, 0.15 M NaCl, pH 7.4) prior to radioimmunoassay.

Preparation of (+) ABA-BSA Conjugates. The ABA-BSA conjugate was prepared by the method of Weiler (104). However, instead of human serum albumin, bovine serum albumin was substituted. The molar coupling ratio was ca. 7 mol ABA bound per mol BSA as determined by isotopic recovery.

Preparation of B-D-glucopyranosyl abscisate. Abscisic acid (±ABA) (Fluka;264.32 mg, 1.0 mmoles) was gradually added and stirred into 2.0 ml of dioxane in a 25 ml round bottom flask at room temperature. After the ABA had completely dissolved, 1.25 ml of absolute ethanol (EtOH) and 3.25 ml of distilled water were gradually added which resulted in a clear yellowish solution. Cesium bicarbonate (194.0 mg, 1.0 mmoles) was added and the pH of the reaction mixture was lowered to 7.0 by adding several milligram more ABA, after which it was gently stirred for 30 minutes. The volume was reduced by one-half via rotary evaporation at 40°C. Absolute EtOH (5 ml) and then benzene (5 ml) were added, and the mixture was reduced to dryness. The resulting yellow crystals were redissolved in EtOH (10 ml) and then benzene (10 ml) was added, and the solution was evaporated to one-third. Benzene (15 ml) was added and the solution was reduced to one-third as before. Benzene was added twice more to the capacity of the 25 ml round bottom

flask and evaporated as described above and the final solution was reduced to dryness. The yellow-white ABA Cs salt crystals were dried in vacuo over P_2O_5 for 3 hours.

One millilitre dry dimethylformamide (DMF) was added to the ABA-Cs salt at room temperature while gently stirring. Acetobromo-a-D-glucose tetraacetate (Sigma; 415.32 mg, 1.01 mmoles) in 0.75 ml dry DMF was added dropwise to the solution. Another 0.25 ml DMF was used to rinse the acetobromo-a-D-glucose tetraacetate from its weighing container and also added to the solution. The resulting solution was stirred overnight at room temperature. The mixture was centrifuged at 7,000 rpm for 15 minutes and the supernatant loaded onto a silica gel G-60 (EM Scientific 230-400 mesh) 1.2 x 100 cm column equilibrated in chloroform:methanol (96:4, v/v). The ABA-GE tAc was eluted with 300 ml of the above solvent mixture into 4 ml fractions number 17-30. Those fractions which showed one spot, Rf 0.824 on TLC (Whatman reverse-phase MKFG silica gel 1 x 3 cm, 200 u thickness) plates developed in chloroform:methanol (96:4, v/v and visualized by UV) were pooled. Other fractions containing ABA-GE tAc and minor contaminants were rechromatographed on a new silica gel G-60 column as described previously after solvent evaporation, and those fractions showing a single spot on TLC were collected again. All fractions containing purified ABA-GE tAc were pooled, and the solvent was evaporated. The white ABA-GE tAc crystals were dried in vacuo over P_2O_5 and stored at $-18^{o}C$ (73-75mp; 247 mg).

The preparation of ABA-GE from ABA-GE tAc was adapted from the procedure of Lehmann et al. (57) and was followed with slight modification in purification. Briefly, 3 g of dehusked, ripe, mature sunflower Helianthus annuus seeds were ground in an ice cold mortar and pestle with 25 ml, 100 mM Na-phosphate buffer (pH 7.0) and sand until homogeneity. The homogenate was centrifuged for 20 minutes at 20,000 rpm which resulted in the formation of 3 layers. The middle layer (17.0 ml) was collected. Eleven millitre of Na-phosphate buffer was slowly added to a solution of ABA-GE tAc (60 mg) in 3.5 ml EtOH, and gently stirred at room temperature. The 17.0 ml of crude sunflower seed enzyme preparation was added and allowed to react for 24 hours. The reaction was stopped with 20 ml of EtOH and the mixture was centrifuged for 20 minutes at 20,000 rpm. The supernatant was collected and reduced in volume to 1 to 2 ml via rotary evaporation, and the concentrated solution was filtered through glass wool and loaded onto a silica gel G-60 column (1.2 x 100 cm) equilibrated in chloroform:methanol:water (75:22:3, v/v). The putative ABA-GE was eluted in 4 ml (fractions 52-65) in the above solvent system. The putative ABA-GE was identified by TLC on the aforementioned Whatman reverse phase silica gel (Rf 0.45; ABA-GE tAc, Rf 0.95) with the above solvent system. The fractions containing ABA-GE were pooled, and the solvent was removed by evaporation. The resultant ABA-GE was dissolved in EtOH (5 ml) and then benzene (10 ml) and these solvents were reduced via rotary evaporation. Approximately 10 ml benzene

was added and reduced 2 more times. Anhydrous methanol was added dropwise to the dry ABA-GE, and the solution was filtered through glass wool to remove any traces of silica gel. Methanol was removed by evaporation under a stream of dry N_2 gas. The residue dried *in vacuo* over P_2O_5 (24.81mg; 55.5%). The identities of ABA-GE tAc and ABA-GE were verified by analysis of micro NMR spectra (Bruker AM-200 spectrometer). The spectra were obtained at 200 mH_z.

Immunization Procedure. An antigen emulsion was prepared by dissolving 1.5 mg lyophilized conjugate in 1.5 ml PBS (0.01 M Na phosphate, 0.15 M NaCl, pH 7.4) and emulsified with 2.0 ml complete Freund's Adjuvant (Difco). Randomly bred New Zealand White rabbits (12 to 16 weeks old) were immunized with a total of 1.0 ml of freshly prepared antigen emulsion by making 2, 0.25 ml intramuscular and 2, 0.25 ml intradermal neck injections. The first collection of antisera began 10 days later. Boosters were administered biweekly and the preparation (45) and titre (114) of antiserum were tested 1 week after each booster until suitable for radioimmunoassay use. Antisera were stored frozen at $-18^{o}C$.

Radioimmunoassay Procedure. The following reactants in order of addition were pipetted into a test-tube in an icebath: 200 ul of buffer (0.01 M Na_1 phosphate, 0.15 M NaCl, pH 7.4), 100 ul of standard or appropriately diluted plant extract, 100 ul of tracer (DL-*cis, trans*-G-^{3}H abscisic acid, 39 Ci/mmol, Amersham; 2000 cpm, 0.0513 pmol), 100 ul of ABA-directed antiserum diluted 1:625 (final assay

dilution). The reactants were swirled by vortexing and incubated in the icebath for 1 hour. Next, 0.5 ml of ice cold saturated $(NH_4)_2SO_4$ was added to each tube to separate free from antibody bound ABA and vortexed. This solution was incubated in the icebath for 30 minutes and then tubes were centrifuged for 30 minutes at 7,000 rpm. From each tube a 0.5 ml aliquot was drawn and pipetted into a scintillation vial and 10 ml of scintillation cocktail added and counted to 1% significance. Blanks for non-specific binding were essentially prepared by procedures described previously (103, 114) where water was substituted for antiserum.

Separation of Free ABA From ABA-conjugates. Thin layer chromatography plates (Slica gel G, Fisher Scientific) were prewashed 2 times in MeOH prior to use. Leaf or bud extracts (50 to 100 ul) were streaked and developed in toluene:ethyl acetate:acetic acid (50:30:4, v/v/v; Rf 0.5 free ABA; 0.0 ABA-GE; 0.33 phaseic acid) (9). The silica gel Rf corresponding to free ABA was collected and extracted in a test-tube with 0.5 ml 95% EtOH. The tubes were centrifuged 30 minutes later at 7,000 rpm for 10 minutes. The supernatant was collected and 100 ul aliquots were added to each replicate radioimmunoassay tube. The EtOH was evaporated under a stream of dry N_2 gas and the radioimmunoassay procedure was follwed as outlined above, except that, 300 ul rather than 200 ul of buffer was used. The recovery of free ABA from 'Tahiti' lime leaf and bud

extracts after thin layer chromatography and radioimmunoassay was ca. 60%.

Results

Reaction Parameters of the ABA Radioimmunoassay. All immunized rabbits responded to the treatment, but in the present study only antisera from 1 rabbit was selected. When incubated under standard assay conditions, the antisera bound 0.40 pmol of ^{3}H (+) ABA at a final dilution of 1:625 and had an affinity binding constant of $Ka=8.5 \times 10^{-12}M$ (88). The binding of ABA to the antiserum was not pH dependent over the range of 4.0 to 10.0 and the reaction between antibody and ABA had come to completion after a period of 1 hour in the icebath. Under the standard assay conditions, 100 ul of 1:625 diluted serum (final assay dilution) bound approximately 50% of tracer. Unspecific binding was less than 3%.

Assay Sensitivity. A typical standard curve in the non-linear plot which was prepared for each day's assay is shown in Figure IV.1 and in the linear, logit/log, transformation in Figure IV.2. The linearity of this plot indicated that reaction equilibrium was attained under the conditions employed. The measuring range of this assay corresponding to the linear range of the logit/log plot was from 25 to 1000 pg/assay and the detection limit at the 95% confidence limit was 25 pg.

Assay Specificity and Accuracy. The specificity of the ABA-directed antibody was tested in several ways. The number of compounds that were either structurally or

Figure IV.1 Standard curve for the (±) ABA radioimmunoassay constructed from n=20 consecutive assays to show day-to-day reproducibility. The bars indicate standard deviations of triplicate samples. Bo=amount of tracer bound in the absence of (±) ABA standard; B=amount of tracer bound in the absence of standard.

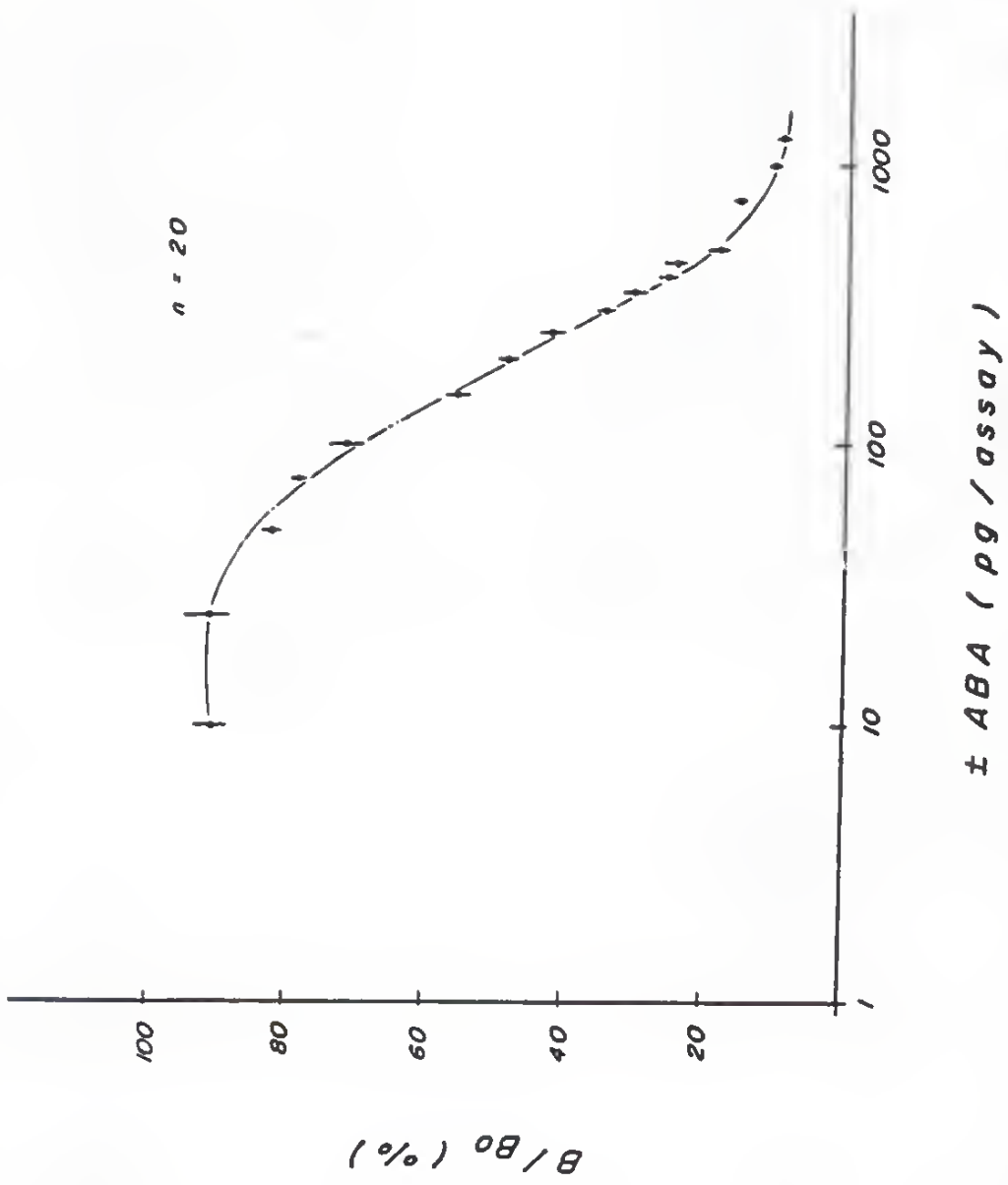
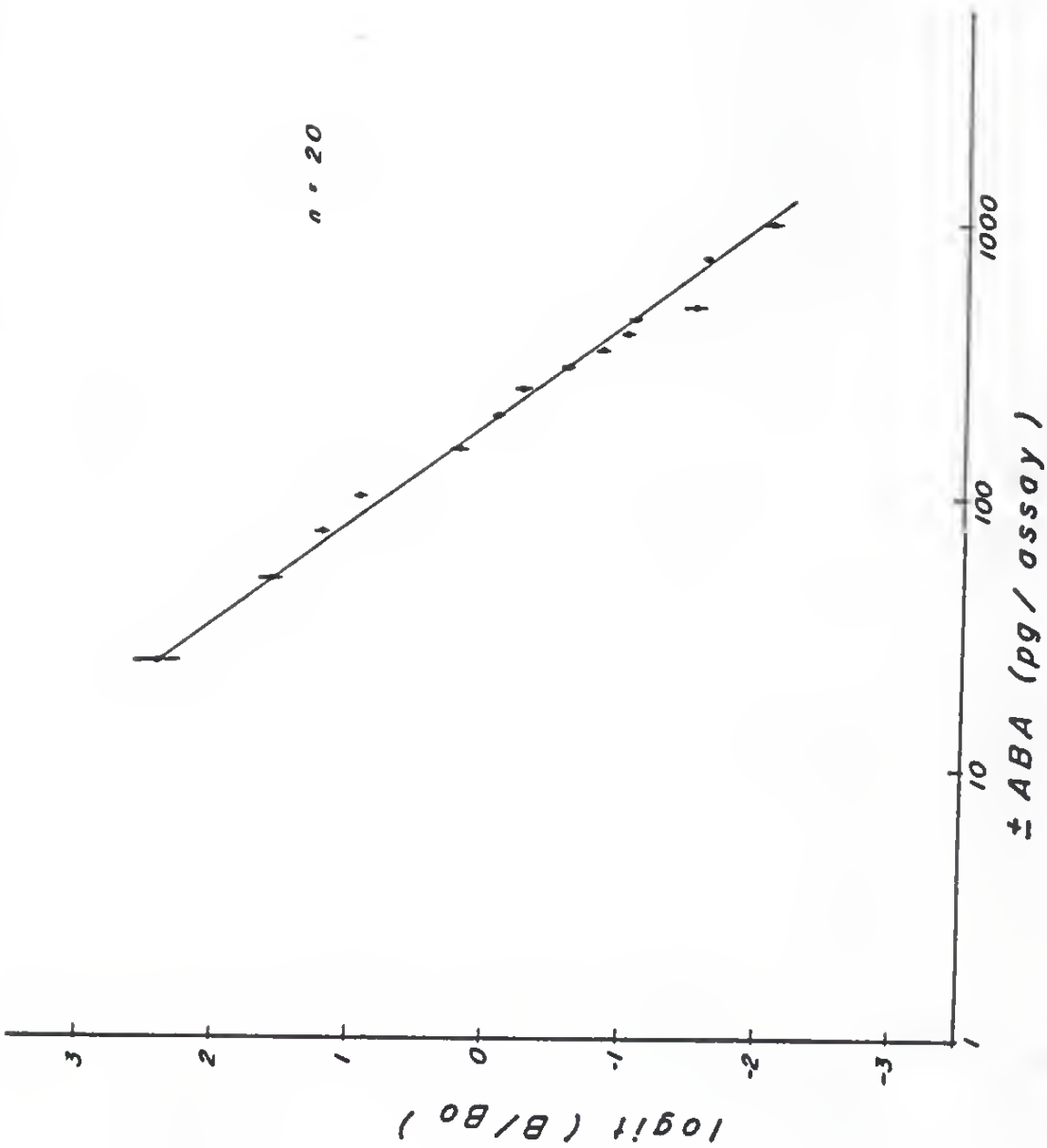

n = 20
100
80
60
40
20
B/Bo (%)
1
10
100
1000
± ABA (pg/assay)

Figure IV.2 Linearized logit-log plot of the standard curve for the (+) ABA radioimmunoassay. logit[(B/Bo)/(100-B/Bo)].


n = 20
logit (B/Bo)
3
2
1
0
-1
-2
-3
1
10
100
1000
± ABA (pg / assay)

physiologically related to ABA were assayed for cross-reactivity with antisera. Cross reactivities were determined as previously described (101, 103, 104, 114). The only compounds which cross-reacted with the ABA-directed antisera were (+) ABA (50.8%) and methyl-(±)-abscisate (75.2%) (Table IV.1). The other compounds tested did not cross-react with the ABA-directed antiserum to any significant extent.

An indication of the specificity of this antisera for ABA is shown in Figure IV.3. Potentially interfering compounds in lime extracts were detected by using extract dilution curves. These curve were found to parallel the standard curve. This parallelism indicates the absence of interfering compounds cross-reacting with the antisera (104) and having different affinity constants as compared to ABA (103, 104). Abscisic acid (50 pg) added to leaf extracts showed a 91% recovery over the dilution range tested and paralleled the standard curve. The assay is precise and reproducible as is evident from Figures IV.1 and IV.2. The standard curve was constructed from n=20 consecutive assays to show day-to-day reproducibility expressed as percent coefficient of variation of 6.1%. Triplicate determinations of unknown samples with readings throughout the measuring range gave coefficients of variation of 5.6% and the complete procedure (including extract processing and immunoassay) is 7.5% for an average sample.

Measurement of Total and Free ABA in 'Tahiti' Lime Leaves. The level of total ABA increased in leaves with

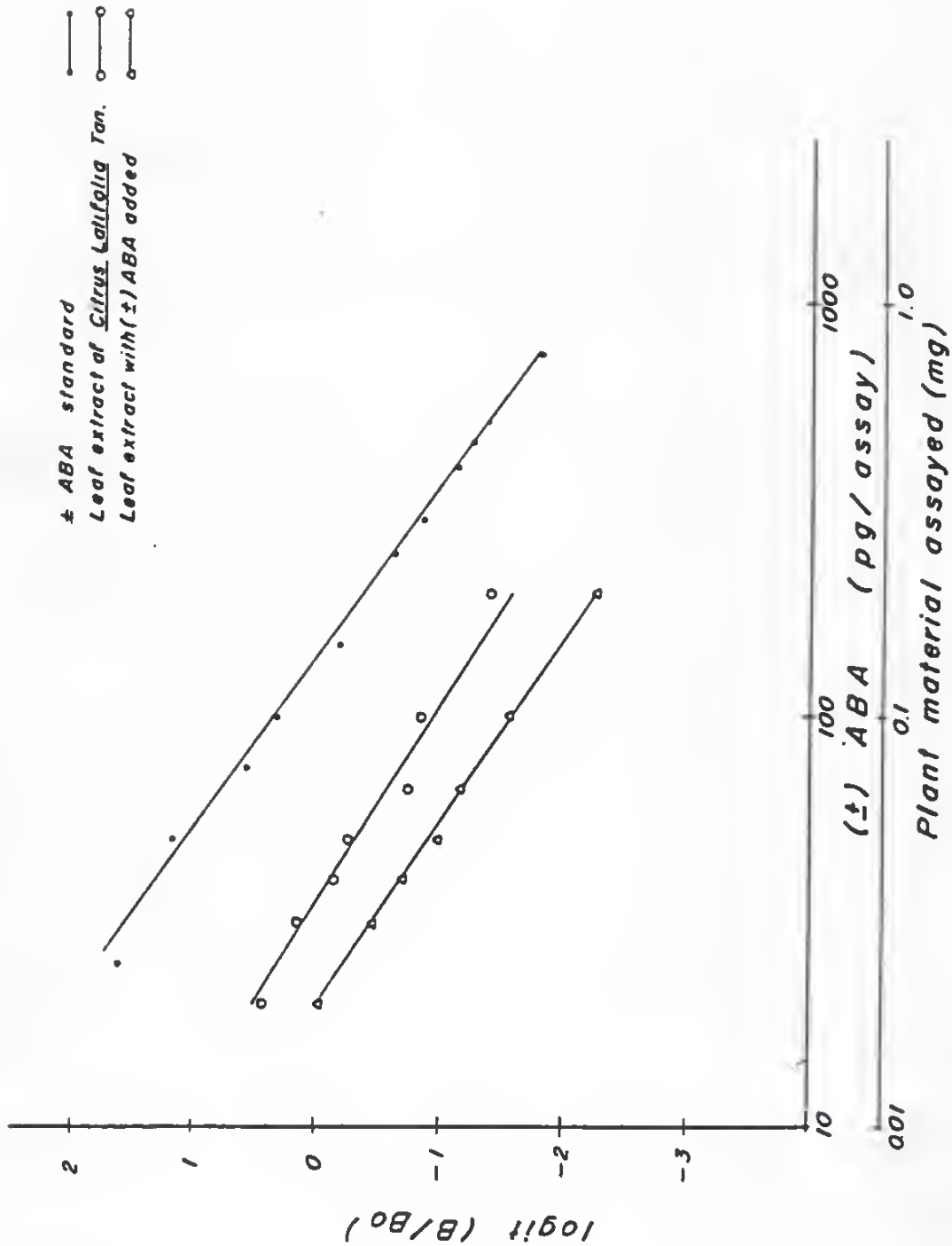
Table IV.1 The specificity of antiserum to abscisic acid.

Compound	pmol Required to Yield B/Bo=50%[z]	Cross Reactivity (%)
(±)-abscisic acid	1.55	100.00
(+)-abscisic acid	3.05	50.80
methyl-(±)-abscisate	2.06	75.20
(±)-abscisyl-(2,3,4,6-O-tetraacetyl)-B-D-glucose ester	15.60	9.90
(±)-B-D-glucopyranosyl abscisate	19.40	8.00
phaseic acid	>178.60*	0.86
dihydrophaseic acid	>355.80*	0.43
xanthoxin	2609.10	0.06

* Highest concentration assayed.

[z] B=% ^{3}H (±) ABA binding in presence of compound; Bo=^{3}H (±) ABA binding in absence of compound.

Figure IV.3 Extract dilution analysis. Diluted leaf extracts of *Citrus latifolia* Tan. contained no (±) ABA or 50 pg/assay (±) ABA.


± ABA standard
Leaf extract of Citrus Latifolia Tan.
Leaf extract with(±) ABA added
logit (B/Bo)
2
1
0
-1
-2
-3
10
100
1000
(±) ABA (pg/assay)
001
0.1
1.0
Plant material assayed (mg)

time elapsed after water stress to a maximum of approximately 2.5 ng/mg frozen tissue (Figure IV.4). Immediately (1 day) after water stress was alleviated by rewatering the soil to field capacity, the level of total ABA fell and continued to fall to a minimum measured at 10 days after alleviating water stress. On the other hand, the level of total ABA was lower in leaves collected from trees growing under low temperature conditions than those leaves grown under water stress conditions. The increasing trend of ABA levels in leaves grown under low temperature conditions was similar to that trend noted for leaves growing under water stress conditions. The amount of ABA measured in leaves growing under low temperature conditions increased as time grown under low temperature increased. However, the increases were not significantly different at each measured time interval.

The levels of free ABA increased in the early part of water stress treatment and reached a maximum (0.24 ng/mg frozen tissue) after 2 weeks (Figure IV.5). After 3 and 4 weeks of stress, however, the level of free ABA decreased. After water stress had been alleviated by rewatering the soil to field capacity, the level of free ABA dropped to non-detectable levels. The levels of free ABA in leaves collected from trees grown under low temperature conditions for 2, 4, and 6 weeks were less than that level measured prior to the beginning of low temperature stress. The free ABA level in leaves of low temperature grown lime trees did not change greatly as a result of time elapsed in the growth

Figure IV.4 Effect of duration of water and low temperature stress on total ABA levels in 'Tahiti' lime leaves as measured by the (±) ABA radioimmunoassay. The bars indicate standard deviations of triplicate samples. The control value at 0 time represents the control level of total ABA throughout the measurement time.

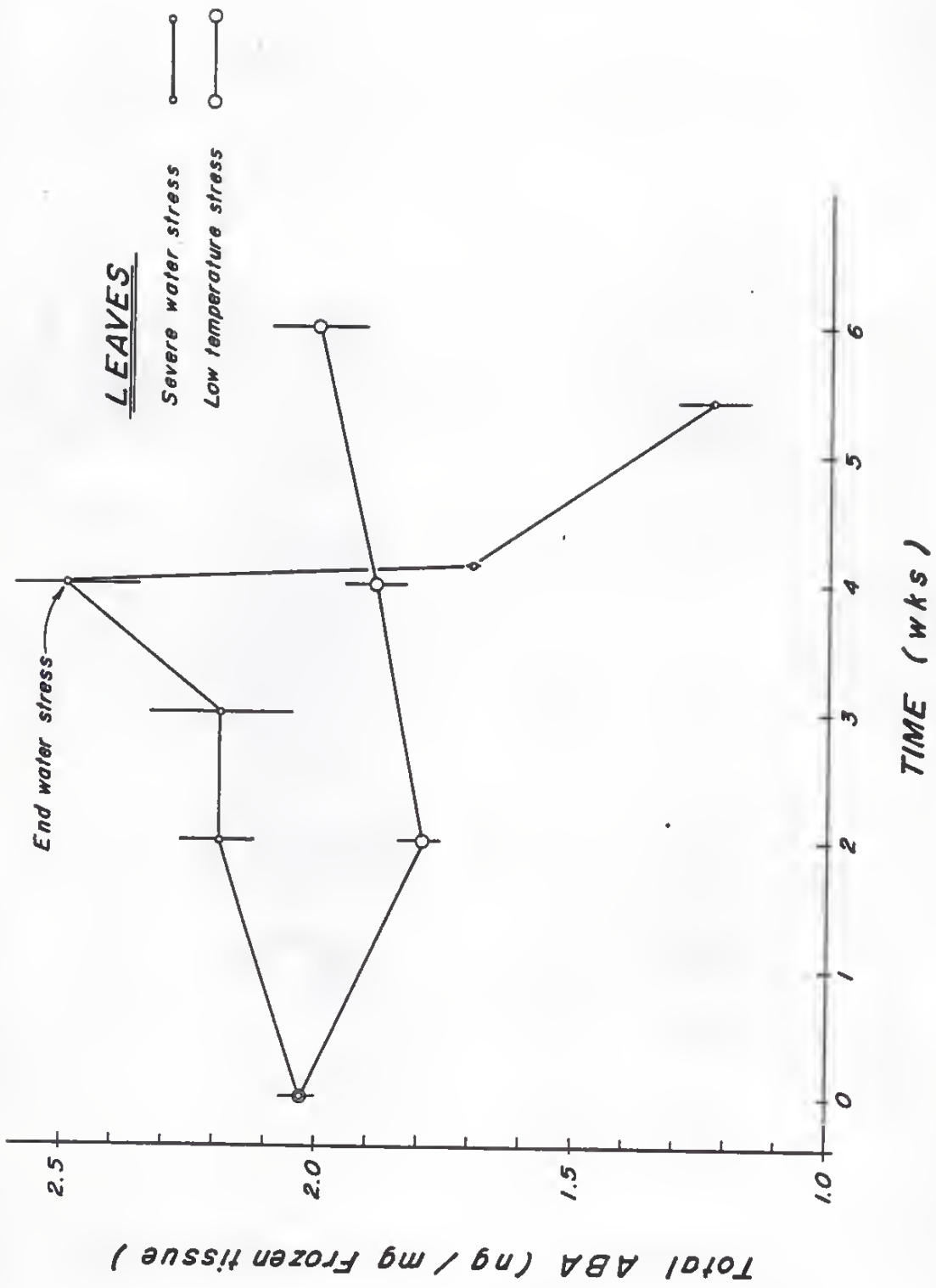
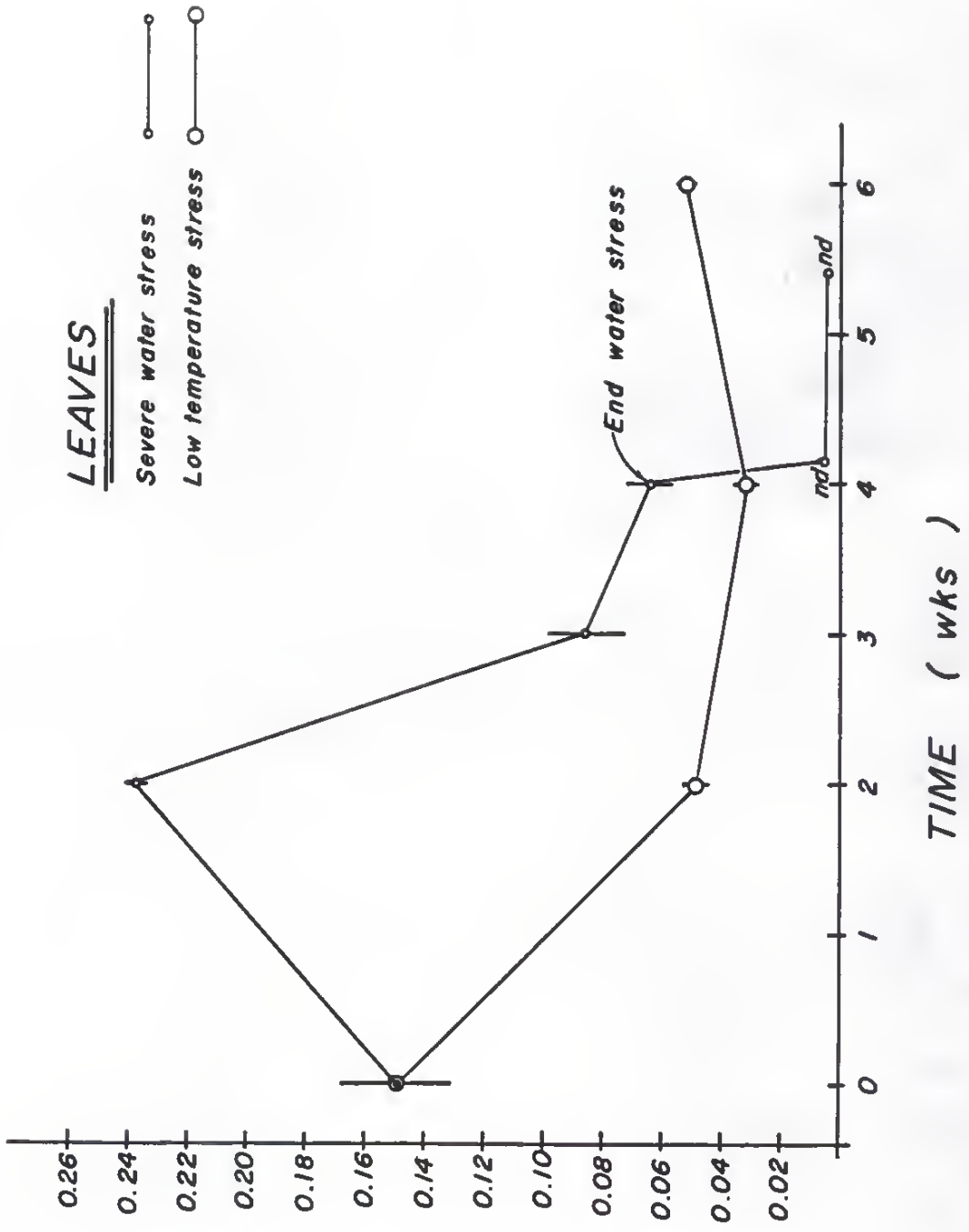

LEAVES
Severe water stress
Low temperature stress
End water stress
Total ABA (ng / mg Frozen tissue)
2.5
2.0
1.5
1.0
0
1
2
3
4
5
6
TIME (wks)

Figure IV.5 Effect of duration of water and low temperature stress on free ABA levels in 'Tahiti' lime leaves as measured by the (+) ABA radioimmunoassay. The bars indicate standard deviations of triplicate samples. The control value at 0 time represents the control levels of free ABA throughout the measurement time. nd = non-detectable levels of free ABA


LEAVES
Severe water stress
Low temperature stress
End water stress
nd
nd
Free ABA (ng / mg Frozen tissue)
TIME (wks)
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6

chamber. The free ABA levels in leaves growing under low temperature conditions were less than those levels measured in water stressed leaves at 2, 3, and 4 weeks.

Measurement of Total and Free ABA in 'Tahiti' Lime Buds. The level of total ABA in lime buds increased as time in which trees were grown under water stress progressed (Figure IV.6). One day after water stress had been alleviated, the total amount of ABA measured in buds decreased to the same level found after 2 weeks of water stress. The levels of total ABA continued to decrease with time elapsed after rewatering and 10 days later the total ABA level was equivalent to that measured in buds prior to the beginning of water stress. The total ABA level measured in buds during the low temperature stress conditions of the growth chamber were less at 2, 4, and 6 weeks than that level measured prior to the beginning of stress. The levels of total ABA measured in buds of trees grown under low temperature stress showed an increasing trend of ABA level against time spent growing in low temperature conditions.

The levels of free ABA in buds decreased as the time trees were maintained under water stress conditions increased (Figure IV.7). The levels of free ABA dropped to non-detectable levels after water stress had been alleviated by rewatering. The free ABA levels measured in buds collected from trees after 2 weeks of low temperature conditions were less than those levels measured prior to the beginning of stress. The levels of free ABA measured in buds at 2 and 4 weeks of low temperature stress were not

Figure IV.6 Effect of duration of water and low temperature stress on total ABA levels in 'Tahiti' lime buds as measured by the (±) ABA radioimmunoassay. The bars indicate standard deviations of triplicate samples. The control value at 0 time represents the control level of free ABA throughout the measurement time.

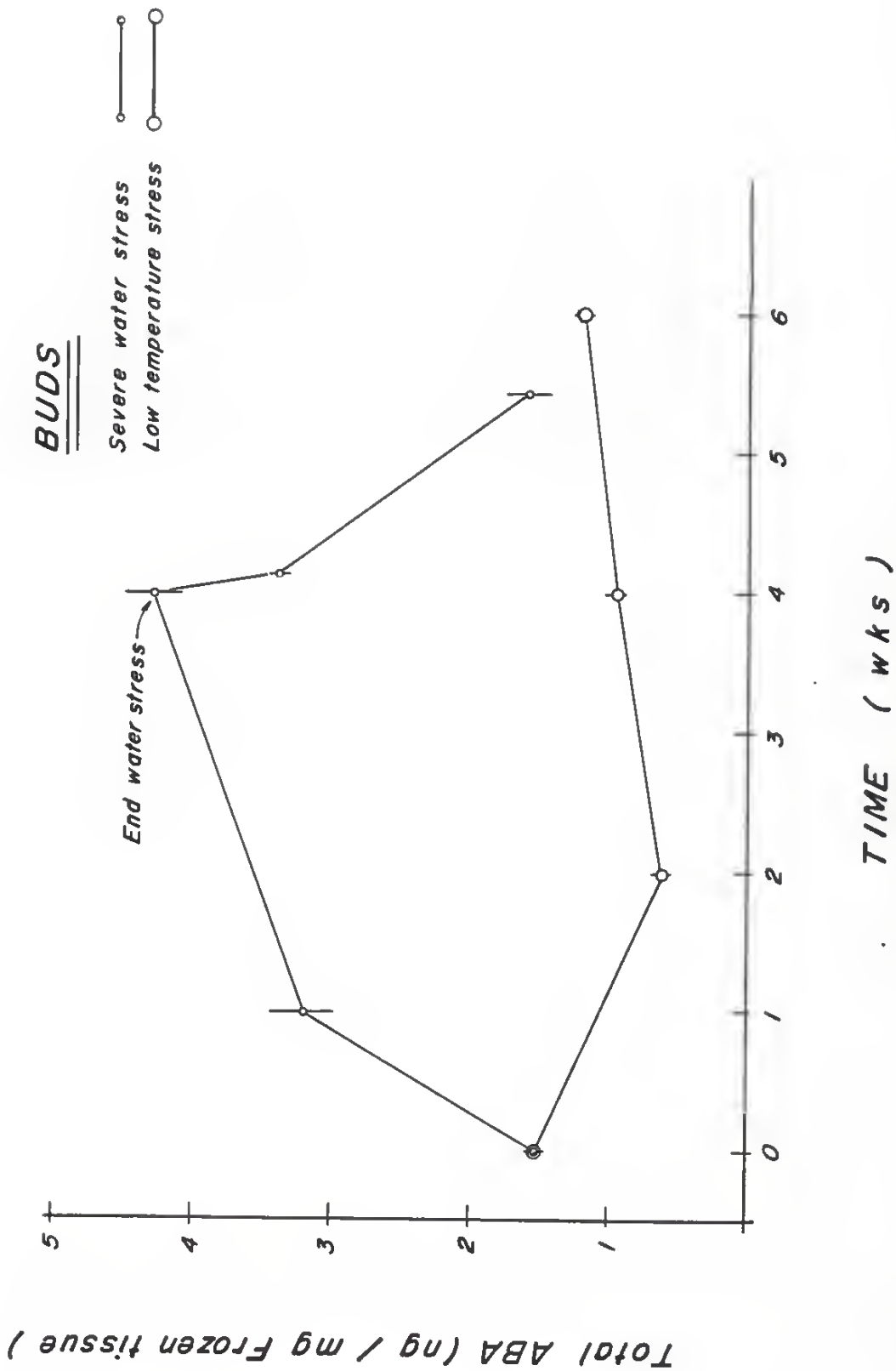
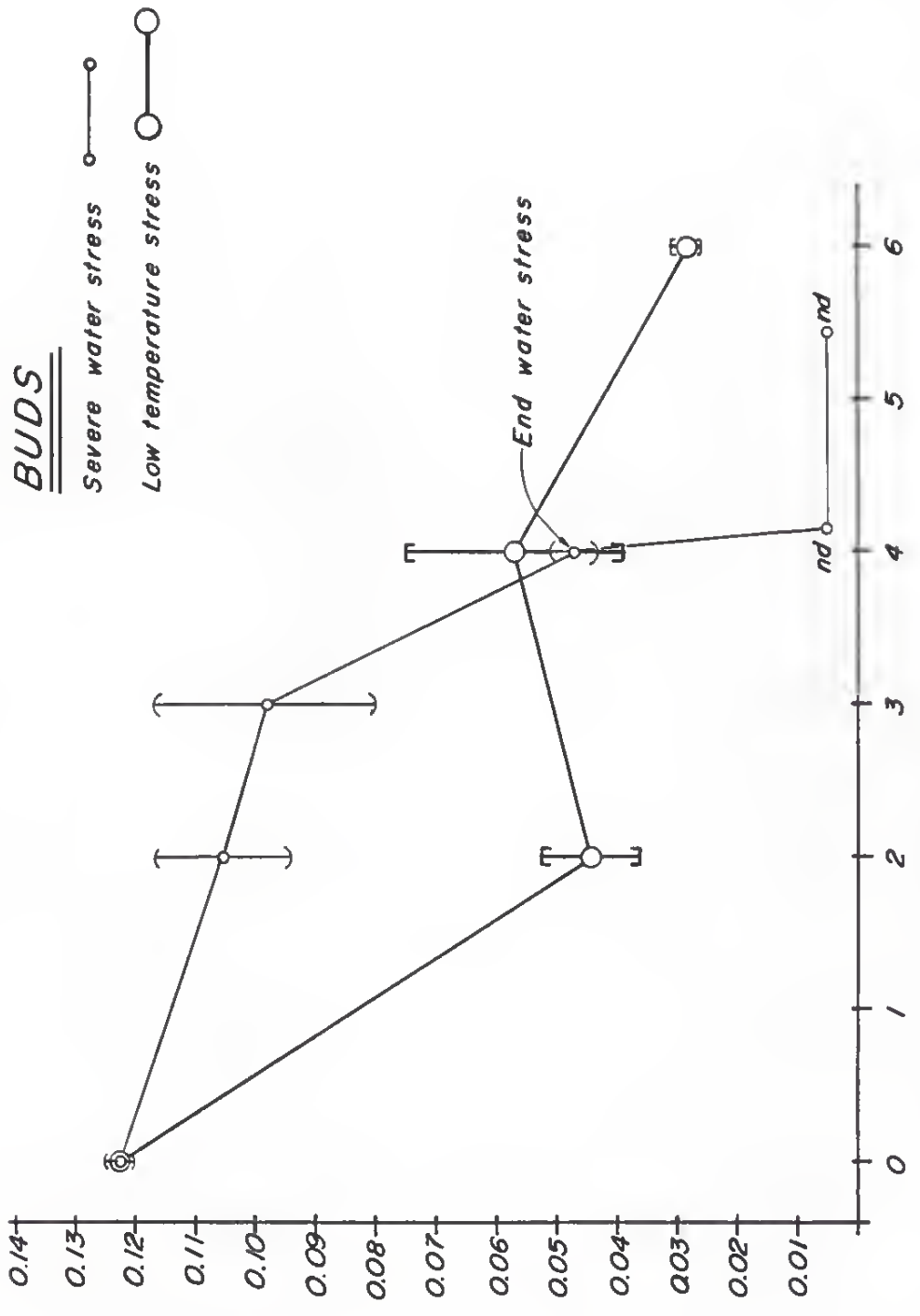

BUDS
Severe water stress
Low temperature stress
End water stress
Total ABA (ng / mg Frozen tissue)
TIME (wks)
0
1
2
3
4
5
6
1
2
3
4
5

Figure IV.7 Effect of duration of water and low temperature stress on free ABA levels in 'Tahiti' lime buds as measured by the (±) ABA radioimmunoassay. The bars indicate standard deviations of triplicate samples. The control value at 0 time represents the control level of the ABA throughout the measurement time. nd = non-detectable levels of free ABA


BUDS
Severe water stress
Low temperature stress
End water stress
nd
nd
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
2
3
4
5
6
Free ABA (ng / mg Frozen tissue)
TIME (wks)

different from one another. ABA levels measured at 6 weeks were slightly less than those measured at 2 and 4 weeks of low temperature stress.

Discussion

Antibodies were obtained that were specific for (±) ABA. These antibodies can be used in a radioimmunoassay which permits the direct quantification of total ABA between 25 and 1000 pg/assay in unpurified extracts from 'Tahiti' lime. The antisera showed cross-reactivity with methyl-(±)-abscisate (75.2%) and (±)-abscisic acid (50.8%) and these results are similar to those reported previously (101, 103, 104). In those previous reports where radioimmunoassays had been developed for (±) ABA coupled through the C 1, COOH (101, 103, 104) functional group, the methyl ester of ABA significantly cross-reacted with the antisera. The (+) enantiomer of abscisic acid was not preferentially bound in those previously reported assays. Rather, the (-) enantiomer of ABA bound more preferentially than the (+) enantiomer. The reason for this is not clear (104). In the case of the assay reported here, no preferential binding of the (-) enantiomer of ABA was apparent as evidenced by 50. 8% cross-reactivity of (+) ABA. Nevertheless, the fact that (+)-abscisic acid is the naturally occurring form of ABA found in plants (119, 120), and only cross-reacted 50% with the antisera, may have decreased the sensitivity of this assay when measuring ABA in plant extracts. In citrus, however, it is not known whether the (+) enantiomer of ABA is found exclusively. Xanthoxin and phaseic acid may occur

in amounts comparable to those of ABA in plants (103). Dihydrophaseic acid may occur in hundredfold higher amounts (100, 101, 103, 119). These compounds did not cross-react with our antiserum to any significant extent and the assay should not be affected at any concentration within the physiological range.

Other potential cross-reactants against this ABA-directed antisera, (±)-abscisyl-(2,3,4,6-O-tetraacetyl)-B-D-glucose ester (ABA-GE-tAc) and (±)-B-D-glucopyranosyl abscisate (ABA-GE) (53, 81), did not cross-react to the same extent as that which had been reported previously for (±) ABA-directed antisera (101, 103, 104). The reason for this is not clear; however, it has been found that antibodies developed by different persons through the same procedures can have different biochemical characteristics (Dr. A. Castro, March 17, 1985, personal communication).

These 2 ABA conjugates, ABA-GE tAc and ABA-GE, were prepared by previously unpublished procedures. The preparation of esters of PGRs has been previously accomplished by reaction of the free acids with O-substituted halocarbohydrates in the presence of triethylamine catalysts (58). However, the use of amines in reaction with alkyl halides can result in racemization of asymmetric carbons. Moreover, the tertiary amine, triethylamine, reacting with an alkyl halide can undergo alkylation to form an amine salt. This reaction side product can reduce the formation of new products and thus reaction yield, thereby requiring more extensive purification. A need exists for a versatile and

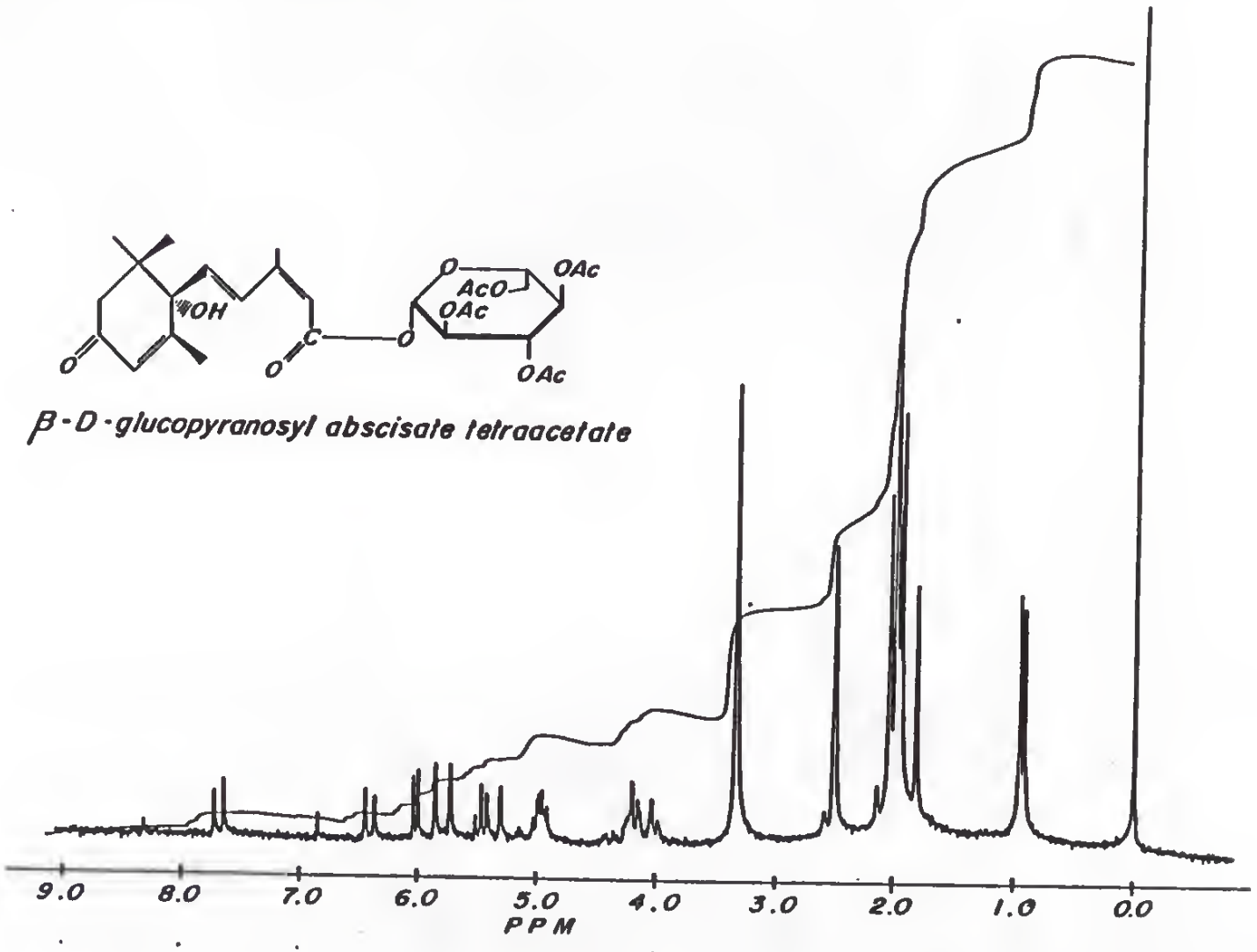
easy procedure to prepare plant growth regulator esters under mild conditions. Cesium carbonate or cesium bicarbonate has been used to form cesium salts of amino acid and peptides (102) which can be reacted with alkyl halides to form esters (44, 102).

The ABA-Cs salt is formed easily by adjusting the reaction mixture to pH 7.0 as had been described for amino acids and peptides (102). The purified ABA-Cs salt reacts upon the addition of acetobromo-a-D-glucose in DMF. Thin layer chromatography after 5, 15, and 30 minutes showed progressively increasing amounts of ABA-GE tAc formed. Yields of ABA-GE tAc (Figure IV.8) and ABA-GE (Figure IV.9) reported were lower than those obtainable via workup of a larger number of fractions because only those fractions showing no evidence of cross contaminating compounds were selected after column chromatography.

The free acid of ABA can be reacted with $CsHCO_3$ to form ABA-Cs salt, which can then be reacted with halocarbohydrates to form esters of ABA. The preparation of ABA-GE by this procedure and purification by silica gel column chromatography are simple and easily scaled up.

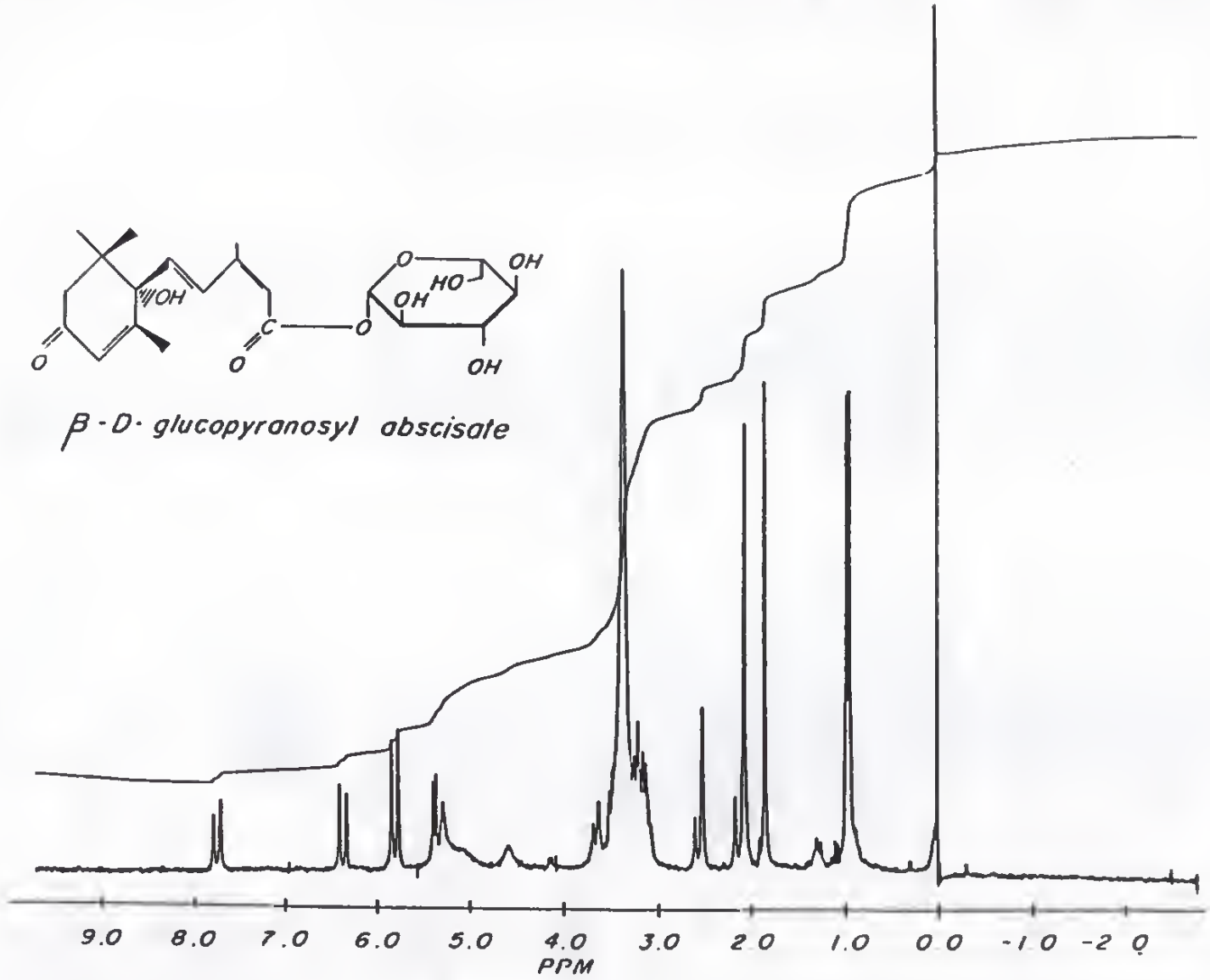
The levels of total ABA measured in water and low temperature stressed leaves were different from one another (Figure IV.4). This observation is understandable in that 2 different stress conditions have been imposed on tree leaves and the ABA levels may change as a result of leaves adapting to different stress conditions. A dramatic change in total ABA levels occurred when water stress was alleviated due to

Figure IV.8 NMR spectra of B-D-glucopyranosyl abscisate tetraacetate in deuterated DMSO.



β-D-glucopyranosyl abscisate tetraacetate

Figure IV.9 NMR spectra of B-D-glucopyranosyl abscisate in deuterated DMSO.



β-D-glucopyranosyl abscisate

rewatering the soil to field capacity. Even as trees continued to recover from stress, the levels of total ABA fell to a minimum of 10 days after water stress had been alleviated. The levels of free ABA paralleled those of total ABA, except those measurements made at the 2 week interval. The level of free ABA was approximately 15 to 35 times less than that of total ABA, which indicated that ABA conjugates, or other cross-reacting compounds previously not described, were responsible for the making up the total ABA measurement. It was unlikely that non-described compounds were cross-reacting with our antisera because extract dilution curves from leaves of 'Tahiti' lime were close to parallel (Figure IV.3) indicating the lack of interference of cross-reacting compounds in the extract with our antibody (104). It is more likely that ABA-conjugates are very high in lime leaves. This was also evident from visualizing TLC plates (silica gel GF 254, Fisher Scientific) under UV after development in toluene:ethyl acetate:acetic acid (50:30:4, v/v/v). Only at the origin could ABA be visualized and that compound visualized corresponded to ABA-GE (9). The presence of bound ABA, which was presumed to be ABA-GE (34), was found to attain 4- to 10-fold higher levels than free ABA in senescent citrus fruit peel. Moreover, the levels of ABA previously reported in citrus flavedo (2.5 ng/mg fresh wt, [34]), and young fruitlets (0.1 ng/mg fresh wt., [29]) as well as previous measurements of total ABA by Walton (101) are quantitively similar to those measurements made in the radioimmunoassay for ABA reported here. Apparently, ABA

levels differ in leaves as a result of the type of stress imposed. The question of whether these changing levels of ABA have any role in citrus flowering cannot be answered by these experiments reported here. However, it was shown in Chapter III that leaves are not required in all cases for the production of flowers in 'Tahiti' lime cuttings.

The levels of total ABA measured in buds during and after water stress of 'Tahiti' lime were greater than those measured at any time during low temperature stress conditions of the growth chamber (Figure IV.6). The levels of free ABA in buds were 10 to 40 times less than total ABA levels, indicating that the levels of ABA-conjugates were changing more so than free ABA. The level of free ABA dropped sharply when measured after 4 weeks of water stress and when measured at 1 and 10 days after alleviating water stress by rewatering (Figure IV.7). This fact would circumstantially suggest that the levels of ABA-conjugates are accumulating during water stress to a more significant extent than under the conditions of low temperature stress. The levels of free ABA decreased after water stress was alleviated suggesting that the decrease in free ABA may be linked to the removal of buds from water stress imposed dormancy. In reports from previous research, there have been mixed results with regard to a generalized role for ABA in bud dormancy and budbreak (87, 104). In species such as Ribes nigrum L. and Fagus sylvatica L. there is a good correlation between the ratio of free and conjugated ABA with the physiological or morphological status of buds

(116), but in species such as Betula pubescens Ehrh., there is not a good correlation. Significant research emphasis has been expended with regard to ABA involvement in bud dormancy and the release of buds from winter dormancy. Seeley and Powell (90) made monthly measurements for 1 full year of free ABA and hydrolyzable ABA in 'Golden Delicious' apple buds. They found that free ABA increased during mid-summer before and after entry into early dormancy and increased to a maximum prior to leaf fall. Thereafter, the free ABA level decreased to a minimum prior to bloom. Hydrolyzable-ABA increased gradually during fall and winter, reached a maximum during the early stages of bud development, and decreased rapidly just prior to full bloom. These researchers concluded from their work and after summarizing the research of others, that ABA has a casual role in the inception of winter dormancy in apple. Abscisic acid was shown to diffuse from apple bud scales (94). Moreover, extracts from bud scales, as well as ABA application to buds which had scales removed, mimicked the inhibitory effect of bud scales on bud growth inhibition during an in vitro experiment with dormant apple buds in branch cuttings (94). In the case of 'Tahiti' lime, there is a good apparent correlation between the levels of free and conjugated ABA and budbreak under conditions of water stress, but not under the conditions of low temperature stress. Apparently, the 2 methods used to induce flowering in lime regulate flowering, but not by altering the levels of ABA in similar fashion.

Total and free ABA in 'Tahiti' lime leaves and buds were measured by radioimmunoassay in these experiments. The levels of ABA-conjugates were present in much higher levels than those of free ABA. In fact, the levels of ABA-conjugates may have been even higher than indicated by RIA because of the lower level of cross reactivity of ABA-GE in our assay than those reported previously (101, 103, 104). The levels of ABA changed in dissimilar fashion when measured in leaves and buds of lime trees grown under either the floral inductive conditions of water or low temperature stress. Therefore, it appears that ABA does not show a consistent pattern of change as a result of floral inductive treatment. Abscisic acid seems to be actively synthesized and metabolized under water stress conditions and the levels of total and free ABA decrease once water stress is alleviated. Abscisic acid may be involved in dormancy and budbreak. The role of ABA in physiological aspects of flowering, budbreak and dormancy of lime is still unclear. Exogenous sprays of ABA as well as more detailed analysis of ABA levels and ABA receptor studies may help elucidate the physiological role for ABA in these processes.

CHAPTER V
DEVELOPMENT OF A GIBBERELLIN RADIOIMMUNOASSAY FOR MEASUREMENT OF GIBBERELLIN LEVELS IN LEAVES AND BUDS OF FLORAL INDUCED 'TAHITI' LIME

Introduction

Evidence from several lines of research suggest that gibberellins (GAs) are the group of plant growth hormones most likely to control flowering in citrus. One line of research showed that GA_3 inhibited flowering in citrus after being applied to citrus as whole tree sprays (5, 17, 37, 74) or as an application to buds (35). These exogenous applications of GA to citrus have shown that GA inhibits flower formation. However, GA applied after a floral inductive water stress period can inhibit flowering (81) and therefore, as a result of GA application, flower buds may never form or revert to vegetative apicies (35, 82). Another line of evidence showed that the use of GA synthesis inhibitors like (2-chloroethyl)trimethyl ammoniumchloride (chlormequat) or succinic acid-2,2-dimethylhydrazide (daminozide) induced large numbers of flowers under certain conditions (73, 74), thereby supporting the likelihood for GA control of flowering.

Even though GA has been found to inhibit flowering when applied to citrus trees, the endogenous GA levels and how they might change as a result of floral inductive treatment are poorly understood in citrus. Gibberellin A_1 has been found in water sprouts of citrus (51). Gibberellin A_1 and GA_9 as well as an unknown gibberellin have been identified

in young 'Washington' navel oranges (52). For the most part, however, only gibberellin-like substances have been isolated from citrus tissues (29, 32, 35, 99, 115). These substances are termed "GA-like" because they exhibit the same activity as GA_3 in GA-sensitive bioassays. Endogenous GA-like compounds were found to be highest in vegetative shoots of citrus (shoots bearing only leaves), next highest in mixed shoots (shoots bearing leaves and flowers) and least in generative shoots (shoots bearing only flowers) (29, 35). These different GA levels correlated with the amount of flowering in each of these 3 shoot types and suggested that the endogenous levels of GA controlled the flowering behavior of citrus.

Gram or killigram quantitites of plant tissue are usually required to make plant hormone measurements because the amounts found in tissues are low and losses are great during extraction and purification. Therefore, to investigate GA changes in small quantities of citrus leaf and especially bud tissue we felt it necessary to develop a GA-sensitive immunoassay. The measurement of GA's by immunoassay has been reported to be sensitive, rapid, reproducible, inexpensive, and require only small quantities of tissue for measurements (3, 4, 113).

In an effort to investigate the role of endogenous gibberellins in citrus flowering, we have induced flowering by low temperature and water stress as previously described in Chapter III. Leaf and bud tissues were collected throughout the stress periods and analyzed for GA in order

to correlate changes in GA levels with the floral inductive treatments.

Materials and Methods

Plant Material and Extraction Procedure. In these experiments, 1- to 2-year-old container grown 'Tahiti' lime (Citrus latifolia Tan.) trees were grown in the greenhouse or growth chamber as described in Chapters III and IV. Five tree replicates/treatment were severely water stressed (at least -3.5 MPa leaf xylem pressure potential, Chapter III) and leaf and bud tissue were collected from trees after 2, 3, and 4 weeks. Water stress was alleviated by watering the container soil to field capacity and leaf and bud tissue were collected 1 and 10 days later. In addition, 5 tree replicates/treatment were placed in the growth chamber under low temperature conditions as described previously (Chapter III). Leaf and bud tissue were collected after 2, 4, and 6 weeks of growth in the chamber. Immediately after each water or low temperature stress interval, leaves or buds from each tree replicate were removed and washed with distilled water, towel dried, pooled together, frozen in liquid N_2, and ground in a mortar with a pestle into a fine powder. The tissue was stored in the freezer at -18°C for less than 90 days until use. The extraction procedure of Weiler (9103) was followed with slight modification. Fifty to 100 mg of frozen powdered tissue was extracted with 90% MeOH, containing 10 mg/l 2,6-di-t-butyl-4-methyl phenol (BHT) (200 ul/mg frozen tissue) for 2 days in the dark at 4°C. The tissue was shaken at regular intervals daily and

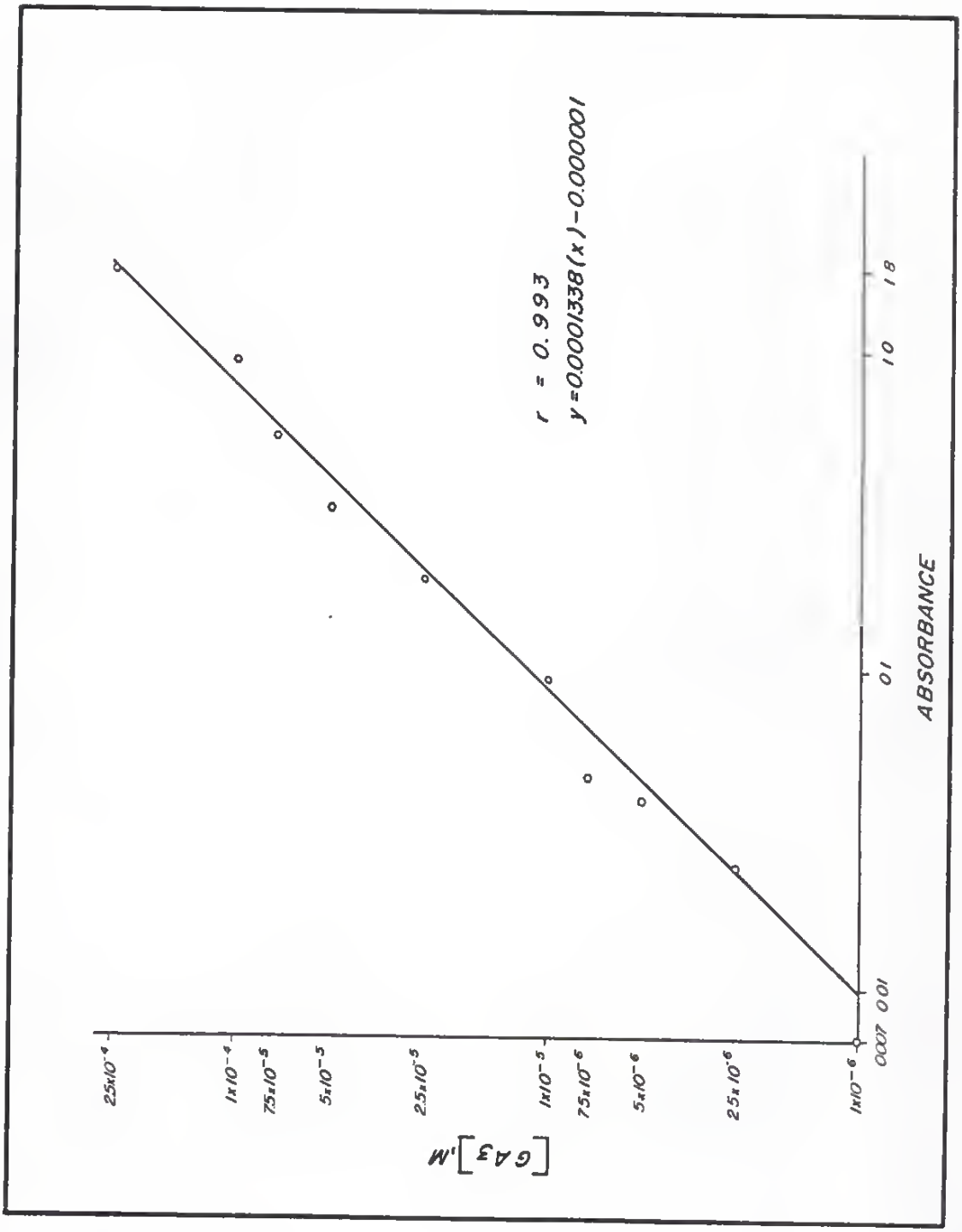
the supernatant collected after centrifugation at 7,000 rpm for 10 minutes. Aliquots of the extract were diluted 1:5 or 1:10 with phosphate buffered saline (PBS, 0.01 M Na phosphate, 0.15 M NaCl, pH 7.4) prior to radioimmunoassay.

Preparation of GA_3-BSA Conjugates by Mixed Anhydride Reaction. The GA conjugate was prepared by the method of Weiler and Wieczorek (113). Thin layer chromatographic analysis (12, 64, 113) revealed the absence of any free uncoupled GA_3 in the diasylate. The coupling of GA_3 to BSA was verified by spectroscopic analysis (21, 22) and the coupling ration was ca. 1.2 to 1.7 mol GA_3 coupled to 1 mol protein. The molar coupling ratio was calculated by developing a standard curve of GA_3 concentration (Figure V.1; GA_3 dissolved in concentrated H_2SO_4) versus the optical density at the peak of the absorption spectrum 415 nm, and then calculating the mol GA_3 coupled to 1 mol protein (21). The GA-BSA conjugate was assayed for the presence of bound GA and no change in the absorption peak maximum was noted; therefore, we concluded that GA was bound to the protein.

Preparation of GA_3-BSA Conjugates by Symmetrical Anhydride Reaction. Gibberellin A_3 was coupled to BSA by the method of Atzorn and Weiler (3, 4). The conjugate was analyzed as above for the presence of free unreacted GA_3 and none was found. The coupling ratio was ca. 5.4 to 7.2 mol GA_3 coupled to 1 mol protein.

Effect of pH on Coupling of GA_3 to BSA via the Mixed Anhydride Reaction. A $0^{o}C$ solution of BSA (840 mg) in 44 ml DMF/water (1:1, v/v) was prepared. This solution was split

Figure V.1 Standard curve of the concentration of GA_3 (M, GA_3 dissolved in concentrated H_2SO_4) versus absorbance (nm) used to calculate molar coupling ratios of GA_3-BSA conjugates.


r = 0.993
y=0.0001338(x)-0.000001
ABSORBANCE
[GA3],M
25x10⁻⁴
1x10⁻⁴
75x10⁻⁵
5x10⁻⁵
25x10⁻⁵
1x10⁻⁵
75x10⁻⁶
5x10⁻⁶
25x10⁻⁶
1x10⁻⁶
0007
001
01
10
18

into 6 equal portions and the pH was adjusted to 6.0, 7.0, 8.0, 9.0, and 10.0. The pH of the final portion was unadjusted. The GA_3 anhydride was prepared on the same molar basis/portion as by the method of Weiler and Wieczorek for preparation of GA_3-BSA conjugates (113). Upon addition of GA_3 anhydride to the BSA mixture, the desired pH was maintained by addition of 1 N NaOH. The BSA solution with an unadjusted pH (control) was reacted by the procedure of Weiler and Wieczorek (113).

Synthesis of GA_3-BSA via Hydroxysuccinimide. Gibberellin A_3 (311.76 mg, 0.9 mmoles) and hydroxysuccinimde (126.5 mg, 1.1 mmoles) were mixed at room temperature (25°C) with 1.8 ml of dry tetrahydrofuran (THF) until completely dissolved. The solution was then cooled to 0°C and stirred at this temperature for 5 minutes. Dicyclohexylcarbodiimide (DCC) (206.0, 1.0 mmoles) was dissolved in 200 ul dry THF. The DCC solution was added to the THF-GA_3 mixture in 2 equal lots. The second lot was added 2 minutes after the first. This mixture was stirred at 0°C for 1 hour and then overnight at 4°C. The mixture was filtered with suction and washed 3 times with dry THF. The filtrate was collected and the THF removed via rotary evaporation. The resultant syrup residue, GA_3 active ester was dissolved in 1.0 ml dioxane and this solution was filtered through glass wool. Another 0.5 ml of dioxane was used to rinse the flask clean of residue and was filtered through the same glass wool. The GA_3 active ester solution was added dropwise in 0.5 ml aliquots to a 0°C solution of BSA (170 mg) in 1.5 ml

water/0.5 ml dioxane. While the mixture was stirred, the pH was adjusted to 7.5 with 1 M $NaHCO_3$ and kept at pH 7.5 for the remainder of these reactions. Thirty minutes later another 0.5 ml of GA_3 active ester was added and the mixture was stirred at 0°C for another 30 minutes. This mixture was stirred for another 4 hours at room temperature. Next, the mixture was cooled to 0°C and the final 0.5 ml of GA_3 active ester was added and stirred for 30 minutes. The mixture was then stirred overnight at room temperature. The GA_3-BSA conjugate was dialyzed for 16 hours against 1 liter dioxane/water (1:4, v/v), and for 5 days against daily changes of 1 liter, pH 7.5 0.1M phosphate buffer. The coupling ratio of GA_3 bound to BSA was ca. 3.1 mol GA_3 to 1 mol protein.

Carbobenzoxychloride Protection of Amino Groups of Amino-n-Caproic Acid. Amino-n-caproic acid (26.34 g, 0.2 moles) was dissolved in 25 ml 4 N NaOH and while stirring was chilled to 5°C on ice. Alternately, 30 ml of 4 N NaOH and 15.65 ml (18.7 gm) of carbobenzoxychloride (CBZ, Chemical Dynamics Corp.) were added to the amino-n-caproic acid mixture in 5 equal portions over a 30 minute period, starting first with CBZ. After each addition, the solution was vigorously shaken and cooled in an ice bath. After all 5 portions had been added to the amino-n-caproic acid solution, the pH was adjusted to 8.0-9.0 with 1 N NaOH and the solution stood overnight at room temperature. This solution was transferred to a separatory funnel. Water (50 ml) and then 100 ml of diethyl ether were added and the

mixture shaken. After shaking, the aqueous layer was separated and saved and another 50 ml of fresh diethyl ether added to the separatory funnel containing the aqueous phase. This mixture was shaken and the aqueous layer recovered as previously described. The aqueous fraction was cooled on ice to 0°C. Once cooled, the aqueous solution was added to a separatory funnel and 150 ml of ethyl acetate was added and the pH adjusted to 2.0 with 6 N HCl. The mixture was shaken and allowed to separate. The aqueous phase was collected again and an equal volume of ethyl acetate was added to the separatory funnel and the mixture shaken. The aqueous phase was drained and the ethyl acetate fractions pooled. The pooled ethyl acetate fractions were washed with 25 ml of cold 1 N HCl and the aqueous portion removed. The ethyl acetate fractions were next washed with 30 ml of water 3 times, and the aqueous portion was removed each time. The ethyl acetate fraction containing CBZ-amino-n-caproic acid was dried over $MgSO_4$ and then filtered with suction. Next, the ethyl acetate was removed via rotary evaporation and a thick oil resulted. To this oily residue, 20 ml of ethyl acetate was added and the solution warmed to 60°C over steam. To this solution, warm (60°C) petroleum ether was added dropwise until the ethyl acetate solution containing CBZ-amino-n-caproic acid became cloudy and then drops of ethyl acetate were added to clear the solution. The solution was cooled to room temperature and once crystals precipitated from the solution it was put on ice. (m.p. 52-54;46.6 gm). Thin layer chromatography of this product on

silica gel G (Analtech, Inc., Newark, DE) plates developed in ethyl acetate:pyridine:acetic acid:water (200:2:6:1, v/v/v/v) revealed one spot, Rf 0.85 (Rf 0.41 amino-n-caproic acid). Spots were visualized by 0.2% ninhydrin in acetone, O-tolidine/KI stain (93).

Preparation of t-butyl ester of N^6-CBZ-amino-n-caproic acid. N^6-CBZ-amino-n-caproic acid (0.15 moles; 39.7915 g) was mixed with 300 ml of methylene chloride and stirred on ice to 0°C in a round bottom flask. To this solution, 1.5 ml of concentrated H_2SO_4 was added and 10 minutes later 100 ml of 99% isobutylene was bubbled into the flask while constantly stirring at 0°C. This clear solution was removed from ice after 15 minutes, capped with a stopper and then allowed to stir at room temperature for 2 days. This solution was recooled to 0°C and then transferred to a separatory funnel and 50 ml of water was added and the mixture shaken vigorously. Next, 25 ml of diethyl ether was added and the mixture shaken. The aqueous layer was removed and the remaining solution was washed with a mixture of 20 ml of saturated NaCl and 50 ml, 1 M $NaHCO_3$. After removing the aqueous layer, the remaining solution was washed 3 times, each with 50 ml saturated NaCl and the aqueous layer removed each time. The remaining solution containing the putative N^6-CBZ-amino-n-caproic acid t-butyl ester was dried over $MgSO_4$ and then filtered. Methylene chloride was removed via rotary evaporation until a thick clear syrup resulted (40.0 g). The product was identified by infrared spectrometry. IR($CHCl_3$) [carbonyl bands of t-butyl ester and

urethane of benzyloxycarbonyl ranging from 1680 to 1750 cm $^{-1}$; and NH band at 3450 cm^{-1}].

Hydrogenolysis of N^6-CBZ-amino-n-caproic acid t-butyl ester. One gram of 10% Pd on carbon was placed in a glass vacuum bottle and 4 ml (48 mmoles) of concentrated HCl was added dropwise to the Pd. Immediately thereafter, the mixture was diluted with 125 ml absolute EtOH. The fully protected N^6-CBZ-amino-n-caproic acid t-butyl ester (16.06 g, 50.0 mmoles) dissolved in 75 ml absolute EtOH was gradually added to the vacuum bottle and the mixture placed on a Parr hydrogenator. Air was removed from the flask and H_2 gas added to 25 psi. The flask was shaken for 2 hours at room temperature. After careful removal from the hydrogenator, approximately 150 ml of the MeOH was added and the mixture filtered quickly under suction to remove Pd catalyst. The filtrate was collected and refiltered through Whatman #1 paper and rinsed with more MeOH to remove all Pd. Methanol was removed via rotary evaporation at 30-35^{o}C. To the residue, 50 ml water was added and the mixture was transferred to a separatory funnel. The mixture was shaken and the aqueous layer removed. The organic fraction was dried over $MgSO_4$, filtered with suction, and the solvent removed via rotary evaporation. Residual traces of solvent were removed from the orange-colored product via vacuum distillation at less than 1 mm Hg. Thin layer chromatography was performed and visualized with ninhydrin, o-tolidine/KI stain as described previously (Rf 0.89 N^6-amino-n-caproic acid t-butyl ester; Rf 0.6 amino-n-caproic acid t-

butyl ester, 2.22 gm, 30.9%). The product was identified and confirmed by infrared spectrometry and nuclear magnetic resonance. IR($CHCl_3$) [carbonyl of t-butyl ester at 1720 cm^{-1} in the absence of benzyloxycarbonyl] NMR ($CDCl_3$) [A singlet ($-O-C(-CH_3)_3$ overlapping with a series of multiplets ($-CH_2-CH_2-CH_2-$) from 1 to 2.2 delta (15H); a multiplet from 2.2 to 2.55 delta (2H;$-CH_2-C=O$); multiplet from 2.6 to 3.0 (2H;$-N-CH_2-$) and a broad band from 3.0 to 3.5 delta (2H;-NH_2)].

Coupling of GA_3 to amino-n-caproic acid t-butyl ester. Amino-n-caproic acid t-butyl ester (221.85 mg, 1.18 mmoles) was added to a round bottom flask by pipette and then 250 ul dry DMF was added. This solution was constantly stirred and GA_3 (346.4 mg, 1.0 mmoles) was slowly added. An additional 0.5 ml of dry DMF was added while the GA_3 was dissolving so that a clear mixture resulted. The mixture was then cooled on ice to 0°C. Dicyclohexylcarbodiimide dissolved in 250 ul dry DMF was added dropwise over a 7 minute period to the GA_3, amino-n-caproic acid t-butyl ester mixture and the reactants were stirred at 0°C for 1 hour. Next, 1-hydroxy-benzotriazole monohydrate (153.14 mg, 1.0 mmole, Aldrich Chemical Co.) dissolved in 250 ul of dry DMF was added over a 1 minute period to the above mixture. This mixture was stirred overnight at 4°C and then centrifuged at 7,000 rpm for 15 minutes. The supernatant was collected into a separatory funnel and 10 ml of ethyl acetate added. The ethyl acetate fraction was washed 4 times, each time with 1 ml of saturated NaCl and the aqueous phase was removed after

each wash. Next, the ethyl acetate fraction was washed 3 times, each time with 1 ml of 1 M citrate buffer, then, washed each of 4 times with 1 ml saturated NaCl, and finally washed 3 times, each time with 1 ml of a 1:1 (v/v) mixture of 0.1 M $NaHCO_3$/saturated NaCl. The aqueous fraction in every case was removed after each wash. The ethyl acetate fraction was collected and dried over $MgSO_4$, filtered with suction, and the solvent evaporated via rotary evaporation and resulted in a gummy, yellow-green product (509.2 mg, 89.6%). This product was redissolved 5 ml ethyl acetate and centrifuged at 7,000 rpm for 10 minutes to remove residual dicyclohexylurea. The supernatant was collected and the solvent evaporated via rotary evaporation until white crystals remained (439.5 mg, 77.3%). Thin layer chromatography (Whatman reverse-phase MKFG silica gel 1 x 3 cm, 200 u thickness) plates developed in benzene:water:acetic acid (9:1:9, v/v/v), and prepared for visualization under UV as described by Cavell et al. (12) and MacMillan and Suter (64) and/or by the ninhydrin, o-tolidine/KI stain, showed Rf 0.71 GA_3-amino-n-caproic acid t-butyl ester. The product was identified by infrared spectrometry. IR ($CHCl_3$)[showed carbonyl bands of amide from 1630 to 1680 cm^{-1}; t-butyl ester bands from 1690-1730 cm^{-1}; lactone bands from 1740 to 1790 cm^{-1}].

Removal of t-butyl ester from GA_3-amino-n-caproic acid. Gibberellin A_3-amino-n-caproic acid t-butyl ester (439.5 mg) was cooled on ice to 0^oC and 1.0 ml of anisole (Aldrich Chemical Co.) was added and the mixture slowly stirred. One

ml of anyhdrous trifluoroacetic acid (CF_3HCO_2) was added to the mixture and it was stirred for 15 minutes at 0°C and then 40 minutes at room temperature. The solvents were evaporated via rotary evaporation and residual solvents were removed with reduced pressure of less than 1.0 mm Hg. The resulting reddish, syrup-like product was dissolved in 1.0 ml MeOH and loaded onto a 90.0 x 2.2 cm LH-20 (Pharmacia) column equilibrated in MeOH. The putative GA_3-amino-n-caproic acid was chromatographed with 200 ml of degassed MeOH and monitored by absorbance at 280 nm. Fractions 16, 17, 18 (4ml) were pooled and the solvent evaporated via rotary evaporation and a rose-colored product remained. Thin layer chromatography plates (Whatman reverse phase, as above) developed in benzene:water:acetic acid (9:1:9, v/v/v) and visualized as described previously, revealed 1 spot Rf 0.68.

Coupling of GA_3-amino-n-caproic acid to BSA. A solution of GA_3-amino-n-caproic acid (341.35 mg, 0.743 mmoles) was dissolved in 0.5 ml DMF and cooled to 0°C. To this solution, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) (427.32 mg, 2.23 mmoles) dissolved in 1.0 ml DMF was added dropwise to the cold GA_3-amino-n-caproic acid solution and stirred constantly for 30 minutes. This solution was added very slowly in drops to a 0°C solution of BSA (300 mg) in 2.0 ml water/0.5 ml DMF, pH 7.7. This mixture was stirred for 30 minutes at 0°C and then overnight at 4°C. The GA_3-amino-n-caproic acid-BSA conjugate was dialyzed against 1 l, DMF/water (1:4, v/v) at 4°C for 1 day

and then against 1 l, 0.1 M Na phosphate buffer pH 7.5 for 4 days. Daily changes of Na phosphate buffer were made. The coupling ratio of GA_3 bound to BSA was ca. approximately 10 mol GA_3 bound to 1 mol protein.

Preparation of Monomeric Adipic Anhydride. Adipic acid (50.0 gm, 0.342 moles) and 150 ml of acetic anhydride were refluxed together for 2 hours. About 100 ml of acetic acid and acetic anhydride were removed under reduced pressure and then an additional 100 ml of acetic anhydride were added. The mixture was refluxed for another 2 hours and then the acetic acid and acetic anhydride were removed under reduced pressure at a temperature slightly below 100°C. The crude product was purified by vacuum distillation and fractions were collected at boiling points between 105 to 125°C at less than 1 mm Hg.

Preparation of Mono-n-succinimidyl Adipate. A solution of 10.0 gm (0.02 moles) of N-hydroxysuccinimate in a minimal amount of DMF and ethyl acetate was added to the crude product (11.8 gm, 0.092 moles, adipic anhydride) in a round bottom flask (200 ml) while stirring. The reaction flask was placed in a desiccator *in vacuo* under P_2O_5. Heat was generated immediately after the addition of hydroxysuccinimide. The reaction was run for 2 days at room temperature. The solvent was removed under reduced pressure in a hot water bath at 40°C. A white, crystalline product was obtained. It was quenched with anhydrous ether, filtered and washed with ether. The crude product was recrystallized with ethyl acetate and a white crystalline

product was obtained (m.p. 99-100°C). A second crystallization from isopropyl alcohol/isopropyl ether yielded white needles (m.p. 101-103°C; Fwt.=243/131; C 10; H 13; N 1; O 6). Calc. C=49.4 H=5.55 N=5.76 O=39.48: Found C=49.15 H=5.41 N=5.91 IR(KBr)[carbonyl band of carboxylic acid at 1700 cm^{-1}; carbonyl band of ONSu at 1742, 1788, 1812 cm^{-1}].

Preparation of GA_4-mono-N-succinimidyl adipate-BSA. A solution of mono-n-succinimidyl adipate (60.75 mg, 0.25 mmoles) and CDI (44.6 mg, 0.275 mmoles) in 1.25 ml dry DMF was cooled to -20°C while gently stirring. To this solution, DCC (51.5 mg, 0.25 mmoles) in 0.75 ml was added dropwise over a 3-minute period. The mixture was stirred at -20°C for 30 minutes and then at -5°C for 30 minutes, and finally at 0°C for an additional 1 hour. To this reaction mixture, we added a solution of GA_4 and 35 ul triethylamine (83.1 mg, 0.25 mmoles) dissolved in 0.5 ml dry DMF with constant stirring at room temperature. The mixture was stirred for 2 hours and then was centrifuged at 7,000 rpm for 10 minutes. The GA_4-active ester was added dropwise over a 30 minute period to BSA (85.0 mg) dissolved in water/DMF (2.0 ml/0.5 ml) at 0°C. This solution was then stirred overnight at 4°C. The following day, again, GA_4 active ester was prepared as described above and added to the same BSA solution which had been cooled to 0°C and had an additional 0.5 ml water added. The GA_4-adipic acid-BSA conjugate was dialyzed against 1 l, DMF/water (1:4, v/v) for 1 day and thereafter against Na phosphate buffer as

previously described for GA_3-amino-n-caproic acid-BSA. The coupling ratio of GA_4 bound to BSA was ca. 59 mol of GA_4 bound to 1 mol protein.

Immunization Procedure. An antigen emulsion was prepared by dissolving 1.5 mg lyophilized conjugate in 1.5 ml PBS (0.01 M Na phosphate, 0.15 M NaCl, pH 7.4) and emulsified with 2.0 ml complete Freund's Adjuvant (Difco). Randomly bred New Zealand white rabbits (12 to 16 weeks old) were immunized with a total of 1.0 ml of freshly prepared antigen emulsion by making 2, 0.25 ml intramuscular and 2, 0.25 ml intradermal neck injections. The injections were made at weekly intervals for 4 weeks and the first collection of antisera began 10 days after the fourth injection. Boosters were administered thereafter biweekly and the preparation (45) and titre (110) of antiserum was tested 1 week after each booster until suitable for radioimmunoassay use. Antisera were stored frozen at $-18^{o}C$.

Radioimmunoassay Procedure. The following reactants in order of addition were pipetted into a test-tube in an icebath: 200 ul of buffer (0.01 M Na phosphate, 0.15 M NaCl, pH 7.4), 100 ul of standard or appropriately diluted plant extract, 100 ul of tracer (1,2(n)-^{3}H gibberellin in A_4, 38.2 Ci/mmol, Amersham; 2,000 cpm, 0.0524 pmol), 100 ul of GA_4-directed antibody diluted 1:125 (final assay dilution). The reactants were swirled by vortexing and incubated in the icebath for 1 hour. Next, 0.5 ml of ice cold saturated $(NH_4)_2SO_4$ was added to each tube to separate free and antibody-bound haptene (24, 114) and the contents

swirled by vortexing. This solution was incubated in the icebath for 30 minutes and then tubes were centrifuged for 30 minutes at 7,000 rpm. From each tube a 0.5 ml aliquot was drawn and pipetted into a scintillation vial and 10 ml of scintillation cocktail added and counted to 1% significance. Blanks for non-specific binding were essentially prepared by procedures described previously (103, 114) where water was substituted for antiserum.

Results

Preparation of GA-BSA Conjugates. The preparation of GA_3-BSA conjugates by the methods of Weiler and Wieczorek (113) (Figure V.2) and Atzorn and Weiler (Figure V.3) (4) yielded relatively low coupling ratios of GA bound to BSA and did not yield antisera directed against GA when screened by the method of Ochterlony (83). We speculated that low molar coupling ratios of GA_3 to BSA were responsible for the lack of GA-directed antibody production and tried to modify the mixed anhydride or symmetrical anhydride conjugation techniques in order to improve the coupling ratios and obtain a GA antibody. One alternative procedure was to adjust the pH of the reaction mixture during the production of GA_3 anhydride. Analysis of these GA_3-BSA conjugates which had been prepared at different pHs yielded no improvement in GA_3 to BSA coupling ratios. In another procedure, the previously prepared GA_3-BSA conjugates were rereacted by the mixed anhydride or symmetrical anhydride procedure as described previously. However, the rereaction of a GA_3-BSA conjugate did not help to improve the molar coupling ratio

Figure V.2 Scheme of synthesis of GA_3-BSA conjugate by the method of Weiler and Wieczorek (113).

Mixed Anhydride

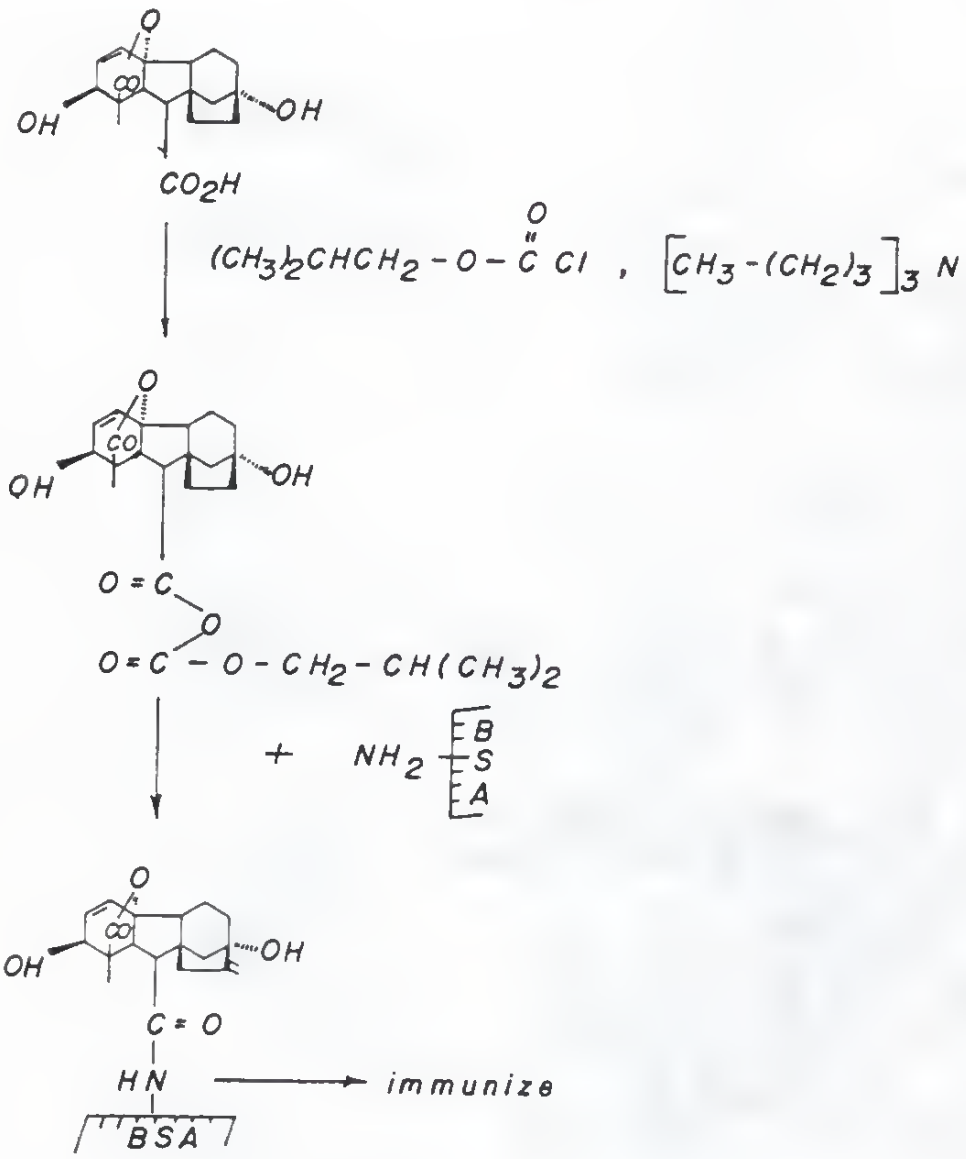
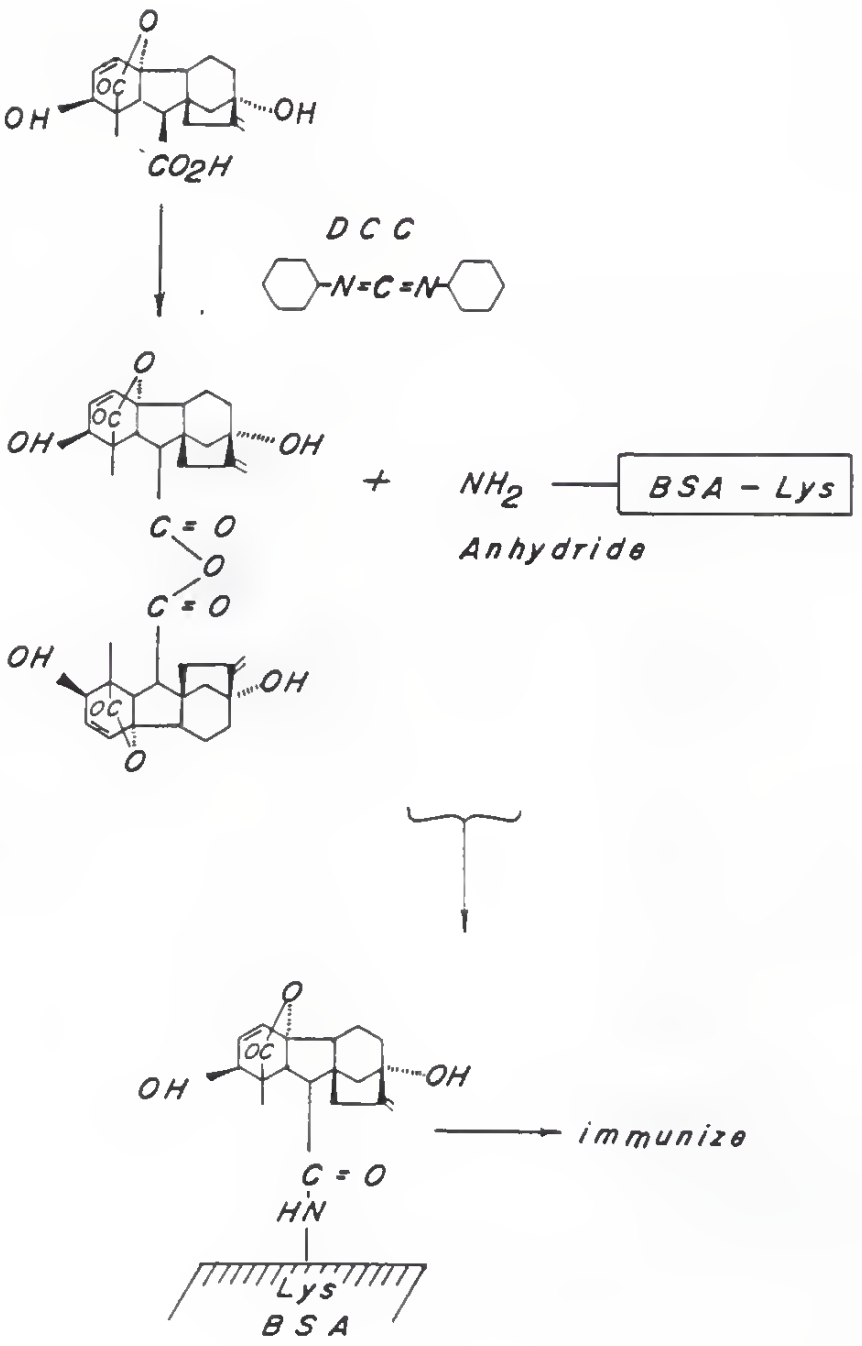


Figure V.3 Scheme of synthesis of GA_3-BSA conjugate by the symmetrical anhydride procedure of Atzorn and Weiler (4).

Symmetrical Anhydride



between GA and BSA. Therefore, we chose to pursue several alternative procedures for coupling GA to BSA (Figures V.4, V.5, V.6). After screening these various antisera collected from rabbits immunized with the various conjugates, we found that the GA_4-adipic acid-BSA conjugate had produced antisera directed against GA_4.

Reaction Parameters of the Radioimmunoassay. The GA_4-adipic acid-BSA conjugate was administered to 3 rabbits and the antisera collected over time until an antibody of appropriate titre was obtained. For the present study, antiserum selected from only one rabbit was used. The maximum tracer binding was obtained at pH 7.4. There was a reduction of binding at pHs lower than 7.0 (4.0, 5.0, 6.0) and at pH 10.0. These observations deviate from those of Weiler and Wieczorek (113) and Atzorn and Weiler (3, 4) who found that inhibition of tracer binding by free gibberellic acid increased at lower pH. Based upon their observation, they decided to use the methyl ester of GA_3 for the remainder of their radioimmunoassay, which increased assay sensitivity. For these studies reported here all GA standards used in radioimmunoassays were non-methylated. Separation of antibody bound from free antigen was achieved by ammonium sulfate precipitation of the immunoglobulin fraction at 50% final saturation (24). The reaction of antibody with haptene was complete after 1 hour and was not significantly different from reactions which continued for 3, 6, and 24 hours. However, the 24 hour reaction time yielded the greatest amount of haptene bound to antibody.

Figure V.4 Scheme of synthesis for the preparation of GA_3-BSA by use of hydroxysuccinimide to create GA_3-active ester.

Synthesis of GA_3 - BSA via Hydroxysuccinimide

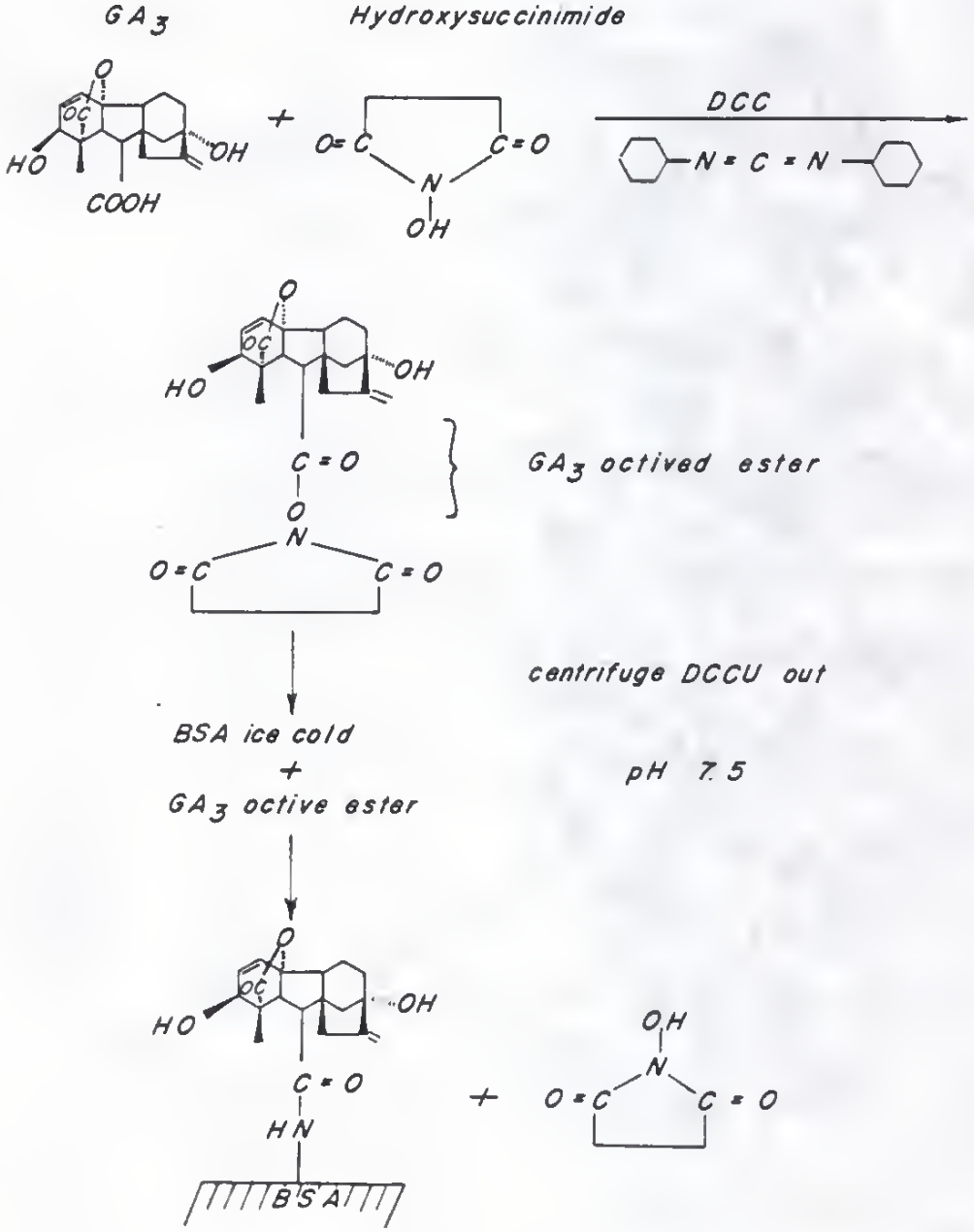


Figure V.5 Scheme of synthesis for the preparation of GA_3-amino-n-caproic acid-BSA.

SYNTHESIS OF GA_3 - ε - AMINO-N-CAPROIC ACID-BSA

GENERAL STRATEGY

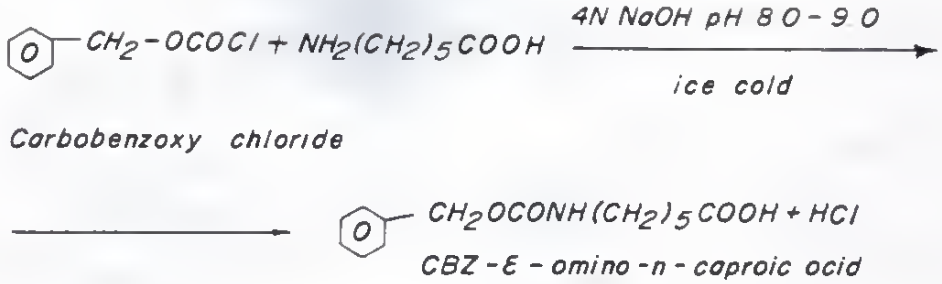
Steps:

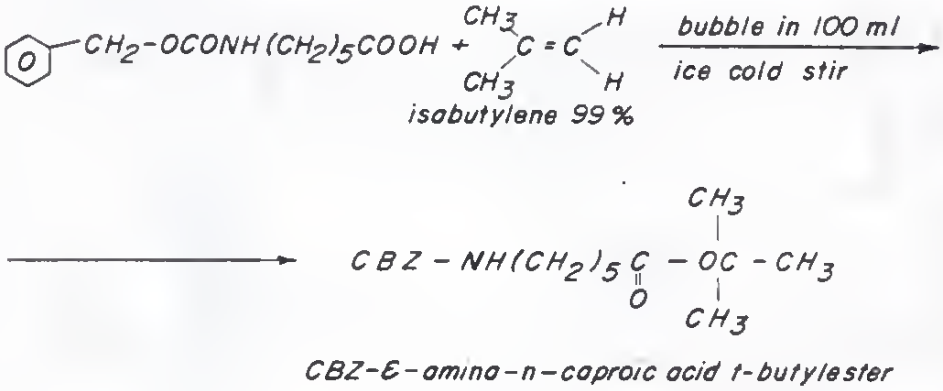
1. Preparation of amino protected(blocked)ε-amino-n-caproic acid.
2. Preparation of carboxy-protected (blocked) CBZ-ε-amino-n-caproic acid

   Result: CBZ (carbobenzoxy chloride) - ε - amino-n-caproic acid-t-butyl ester.
3. Hydrogenolysis (deprotection) of CBZ from CBZ-ε-amino-n-caproic acid-t-BOC.
4. Coupling of GA_3 - COOH to NH_2 - ε - omino-n-coproic acid t-BOC.
5. Deprotection of GA_3 - ε - amino-n-caproic acid t-butyl ester (removal of t-BOC)
6. Coupling of GA_3 - ε - omino-n-caproic acid to bovine serum albumin.

Figure V.5 (continued)

STEP:

1. 
CH2-OCOCl + NH2(CH2)5COOH
4N NaOH pH 8.0-9.0
ice cold
Carbobenzoxy chloride
CH2OCONH(CH2)5COOH + HCl
CBZ-ε-amino-n-caproic acid

2. 
CH2-OCONH(CH2)5COOH +
isobutylene 99%
bubble in 100 ml
ice cold stir
CBZ-NH(CH2)5 C(=O)-O-C(CH3)3
CBZ-ε-amino-n-caproic acid t-butylester

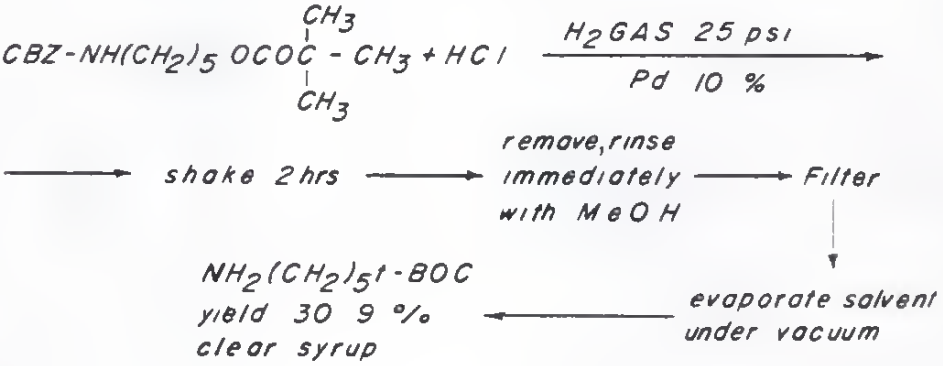
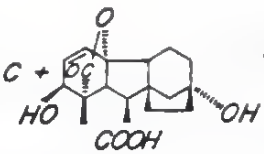
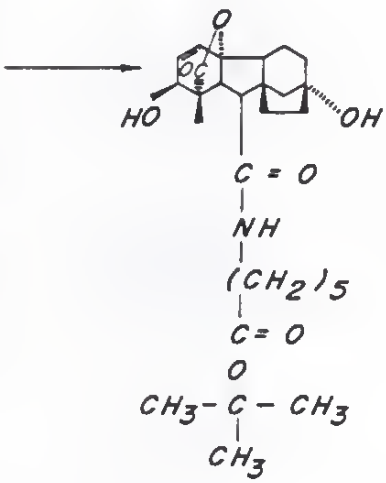
3. 
CBZ-NH(CH2)5 OCOC(CH3)3 + HCl
H2 GAS 25 psi
Pd 10 %
shake 2 hrs
remove, rinse immediately with MeOH
Filter
evaporate solvent under vacuum
NH2(CH2)5t-BOC
yield 30 9 %
clear syrup

Figure V.5 (continued)

4. $NH_2(CH_2)_5$ t-BOC +  —(1-hydroxybenzotriazole, DCC)→

GA_3

—(Remove DCCU)→ wash with NaCl—Citrate—NaCl—NaBicarbonate 3x/ea.

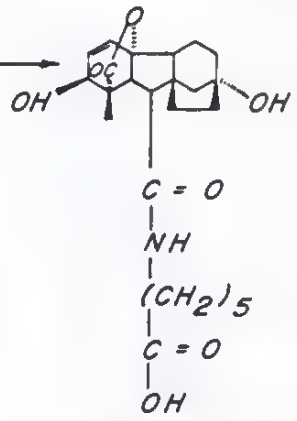


GA_3-ε-amino-n-coproic acid-t-BOC

Figure V.5 (continued)

5. GA_3-ε-amino-n-capraic acid-t-BOC + CF_3CO_2H (Trifluoroacetic Acid)

Anisole, ice, evaporate solvent → Gel filtration LH-20 Pharmacia →



GA_3-ε-omina-n-caproic acid

Figure V.5 (continued)

6. GA_3 - ε - amino - n - caproic acid + 68,000 MW BSA ($(CH_2)_4$, NH_2) — EDC, pH 7.5, H_2O / DMF, ice cold, overnight → dialysis 4 days →

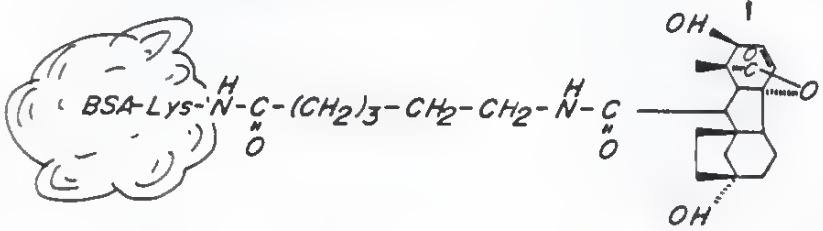
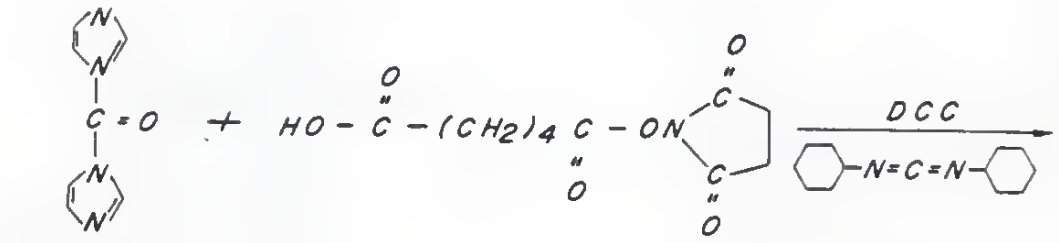


Figure V.6 Scheme of synthesis for the preparation of GA_4-amino-n-caproic acid-BSA.

Synthesis of GA_4 - BSA



1,1'- carbanyl - diimidazole

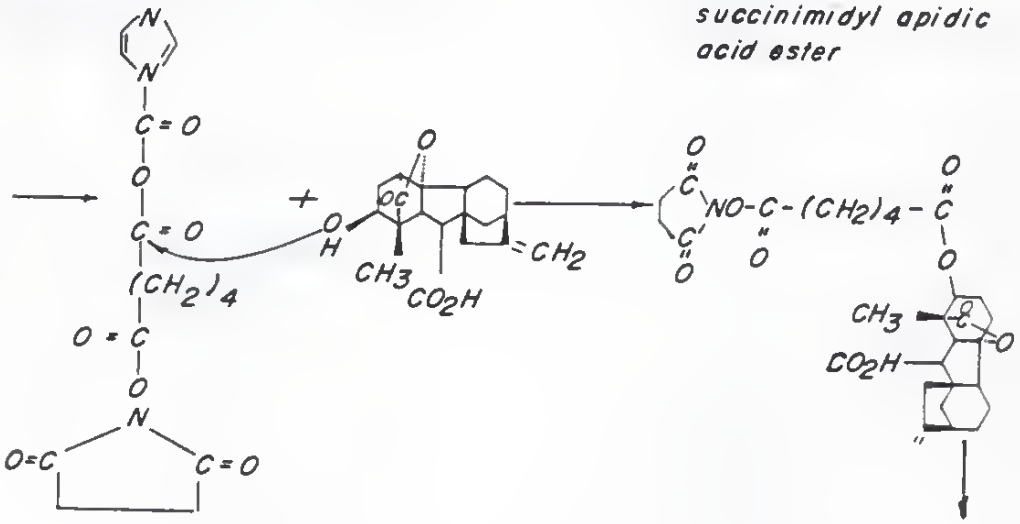
mono - N - succinimidyl adipic acid

helps activate COOH on adipic acid for CDI coupling

nucleophillic attack

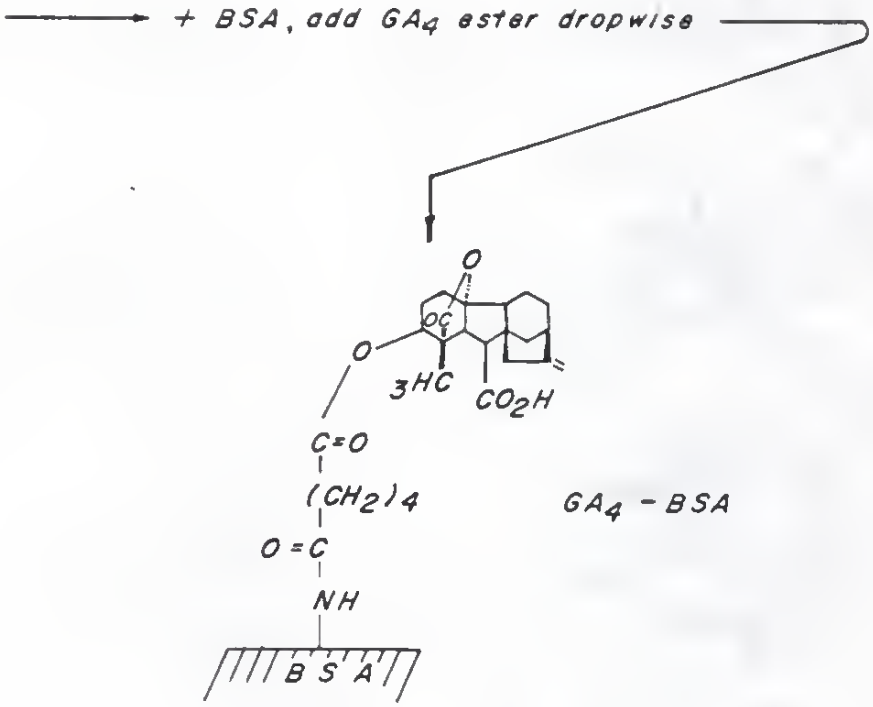
GA_4

GA_4 - mono - n - succinimidyl apidic acid ester



Continue next Page

Figure V.6 (continued)



GA_4 - BSA

Under the standard assay conditions, 100 ul of 1:125 diluted serum (final assay dilution) bound approximately 50% of tracer. Unspecific binding was less than 3%.

From a Scatchard (88) analysis with increasing amounts of tracer, the 1:125 diluted antisera bound 0.3 pmol of GA_4 and a maximum affinity binding constant calculated at Ka=6.4 x 10^{-12} M.

Assay Sensitivity. A typical standard curve in the non-linear plot which was prepared for each day's assay is shown in Figure V.7 and in the linear logit/log transformation in Figure V.8. The linearity of this plot indicated that reaction equilibrium was attained under the conditions employed. The measuring range of this assay corresponding to the linear range of the logit/log plot was from 100 to 1100 pg GA_4 and its detection limit at the 95% confidence limit was 25 pg.

Assay Specificity and Accuracy. The specificity of the GA_4 antibody was tested in several ways. A number of GAs were tested for their ability to cross-react with the antiserum Table V.1. From this table it is evident that GA_7 and GA_9 cross-reacted with the antiserum to a larger extent than GA_4. Gibberellin A_1 was the only other GA tested which cross reacted with the antisera to any significant extent. Apparently, the antisera is specific for GAs with similar structure to GA_4. The double bond between C 1 and C 2 of the GA_7, A gibberellane ring, did not reduce GA_7's specificity (533.0%) for the antiserum. In the case of GA_9, no hydroxylation of C 3 and C 13 improved GA_9's cross-

Figure V.7 Standard curve for the GA_4 radioimmunoassay constructed from n=20 consecutive assays to show day-to-day reproducibility. The bars indicate standard deviations of triplicate samples. Bo=amount of tracer bound in the absence of GA_4 standard; B=amount of tracer bound in the presence of standard.

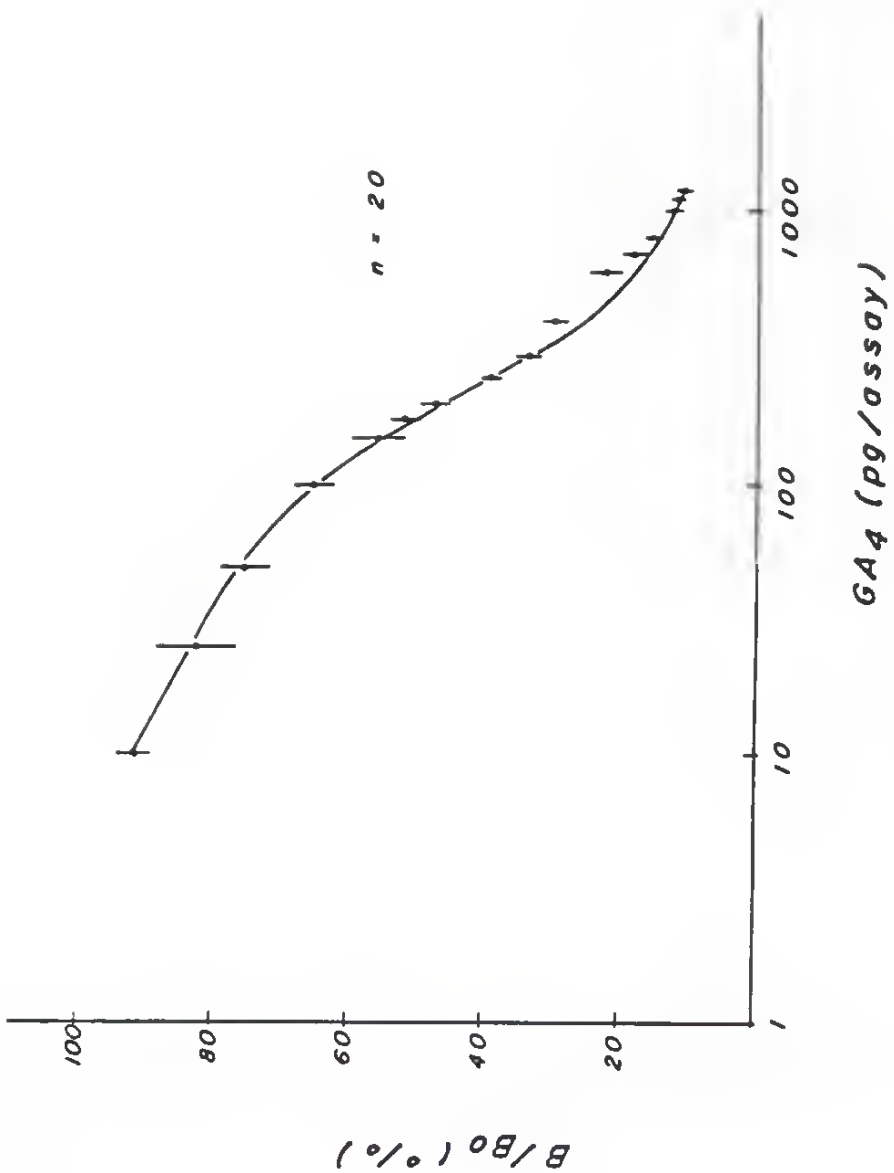

n = 20
B/Bo (%)
100
80
60
40
20
1
10
100
1000
GA4 (pg/assay)

Figure V.8 Linearized logit-log plot of the standard curve for the GA_4 radioimmunoassay. logit (B/Bo)=ln[(B/Bo)/(100-B/Bo)].

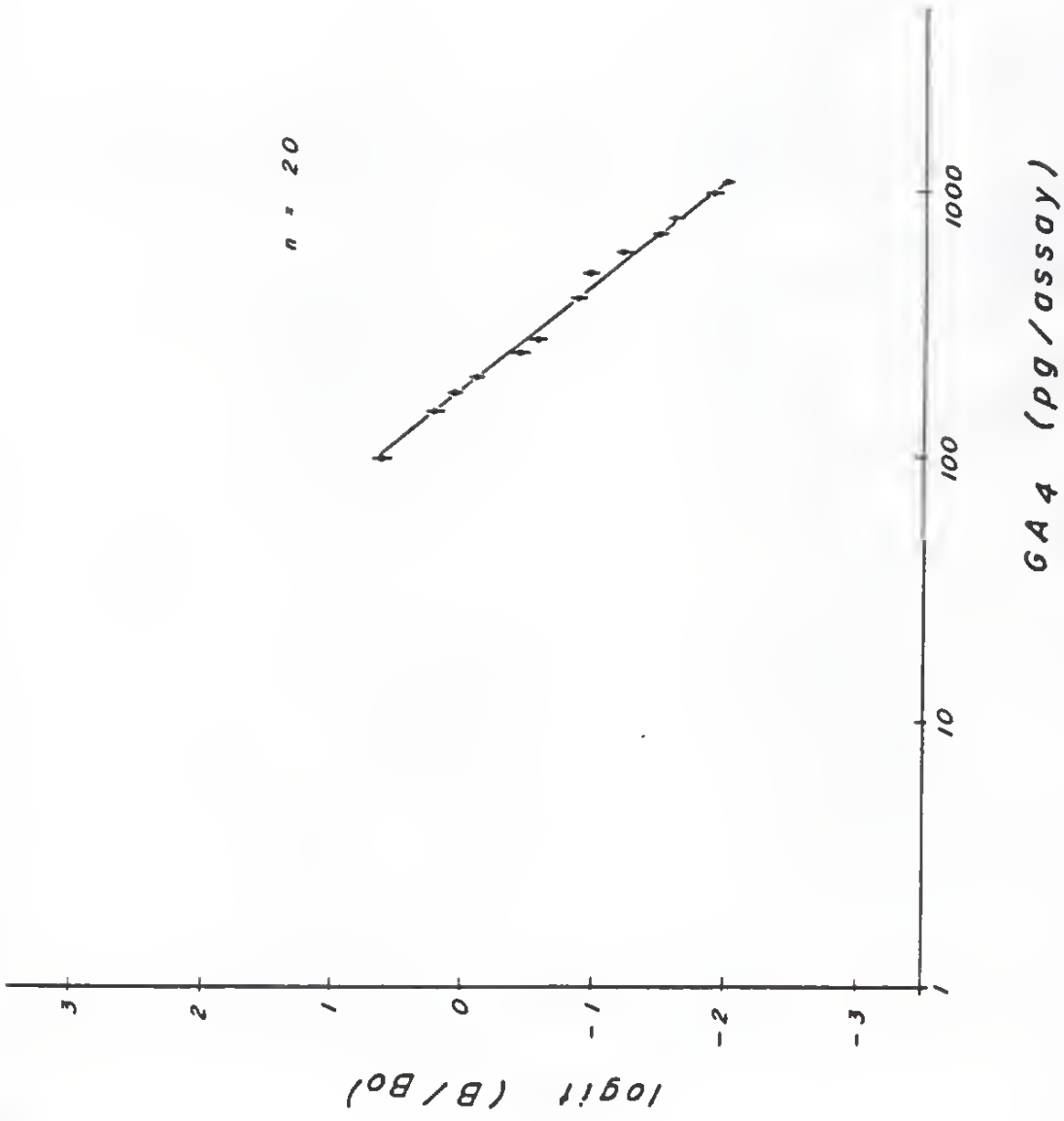

n = 20
logit (B/Bo)
3
2
1
0
-1
-2
-3
1
10
100
1000
GA4 (pg/assay)

Table V.1 The specificity of antiserum to gibberellins.

Compound	pmol Required to Yield B/Bo=50%[z]	Cross Reactivity (%)
GA_1	1.45	66.6
GA_3	11.85	8.1
GA_4	0.96	100.0
GA_5	13.00	7.4
GA_7	0.18	533.0
GA_9	0.20	480.0
GA_{13}	266.50	0.4
GA_{14}	28.70	3.3
GA_{20}	14.70	6.5

[z] B=% $[3_H]$ GA_4 binding in presence of compound; Bo=% $[3_H]$ GA_4 binding in absence of compound.

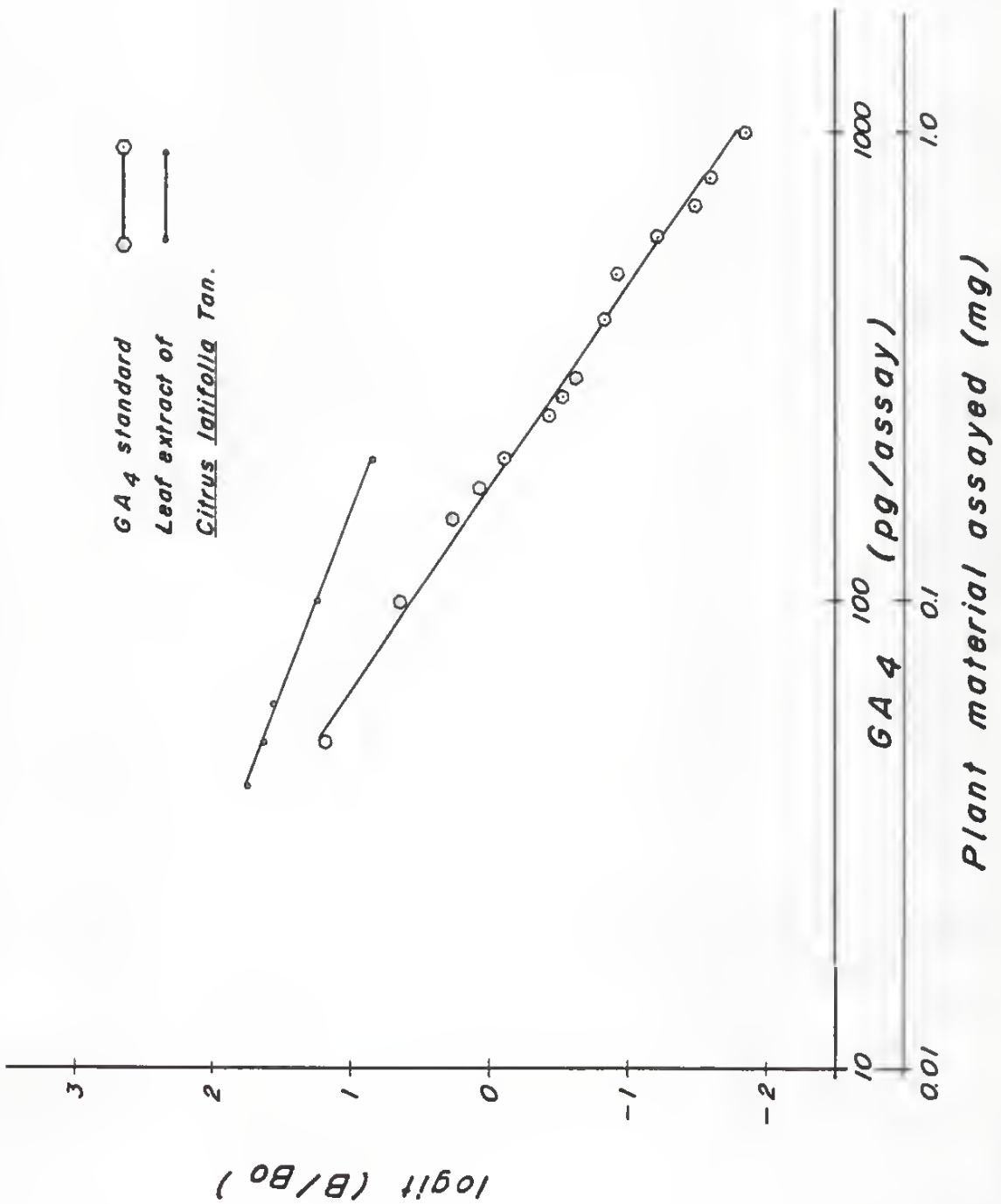
reactivity (480.0%) with the antiserum. Gibberellin A_1, which is identical to GA_9 except that there is no hydroxylation at C 3 or C 13, cross-reacted less (66%). The lack of GA specificity of this antibody indicated that the antibody was a non-specific, general GA antibody. This result differs from those reported by Weiler and Wieczorek (113) and Atzorn and Weiler (3, 4) where they reported much better selectivity of their GA antisera, except for GA_1 antisera which showed cross-reactivity against GA_3, GA_4, and GA_7, but to a lesser extent than reported here.

Another indication of the lack of specificity of this antisera is demonstrated in Figure V.9. The logit/log transformed data of 'Tahiti' lime leaf extract dilution was linear, but intersected with the GA_4 standard curve. Gibberellin A_4 (50 pg) added to extracts showed a 90% recovery, however, the extract dilution range of GA sensitivity was narrow and GA_4 became non-detectable at dilutions greater than 1:50.

The assay is precise as is evident from the standard curve Figure V.7 and V.8. Triplicate samples (standards) show coefficients with a variation of 1.0% and the complete procedure (including extract processing and immunoassay) is 8% for an average sample.

Gibberellin Levels in Water and Low Temperature Stressed 'Tahiti' Lime Leaves. Leaves were collected from 'Tahiti' lime trees within a floral inductive period of severe water stress (-3.5 MPa, Chapter III) or low temperature stress. Under water stress conditions, the GA

Figure V.9 Extract dilution analysis.


GA4 standard
Leaf extract of
Citrus latifolia Tan.
logit (B/Bo)
3
2
1
0
-1
-2
10
1.0
0.01
GA4 100 (pg/assay) 0.1 1000
Plant material assayed (mg)

level rose to a maximum of 0.4 ng/mg frozen tissue after the first week of water stress (Figure V.10). The GA levels dropped and were not different from one another, after 3 and 4 weeks of water stress, but these levels were greater than those measured prior to the beginning of water stress. One day after water stress had been alleviated in these trees by adding water back to the soil, the GA level dropped to 0.071 ng/mg frozen tissue, the lowest level measured. Ten days after water stress had been alleviated, the GA level rose to a slightly higher point than that measured prior to the beginning of water stress.

Gibberellin levels in leaves dropped to non-detectable levels 2 weeks after the floral inductive conditions of low temperature stress began. After 4 weeks of low temperature stress, however, the GA level had increased and reached approximately that GA level measured after 4 weeks of severe water stress. The GA levels measured in leaves after 6 weeks of low temperature stress treatment did not differ from those measured after 4 weeks.

Gibberellin Levels in Water and Low Temperature Stressed 'Tahiti' Lime Buds. Gibberellin levels measured in buds of water and low temperature stressed lime buds were similar in magnitude to those measured in leaves (Figures V.11 and V.10), however, the pattern of change was dissimilar. The GA level measured in buds after 1 week of water stress was not different from that measured prior to the beginning of water stress. The GA level began to decline 3 weeks after water stress and reached a minimum

Figure V.10 Effect of time of water and low temperature stress on GA levels in 'Tahiti' lime leaves as measured by the GA_4 radioimmunoassay. The bars indicate standard deviations of triplicate samples. nd=non-detectable levels of GA. The control value at 0 time represents the control level of GA throughout the measurement time.

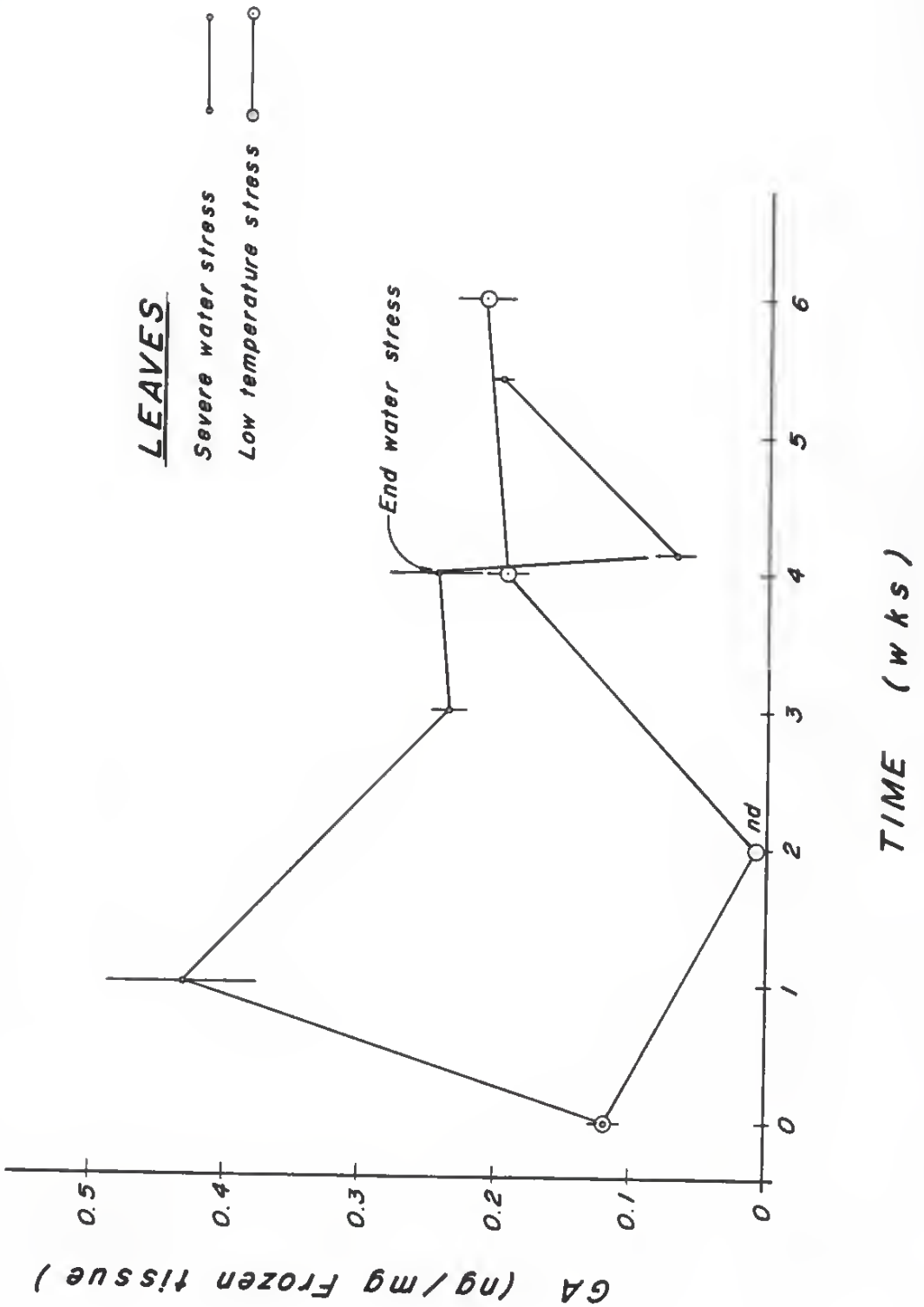
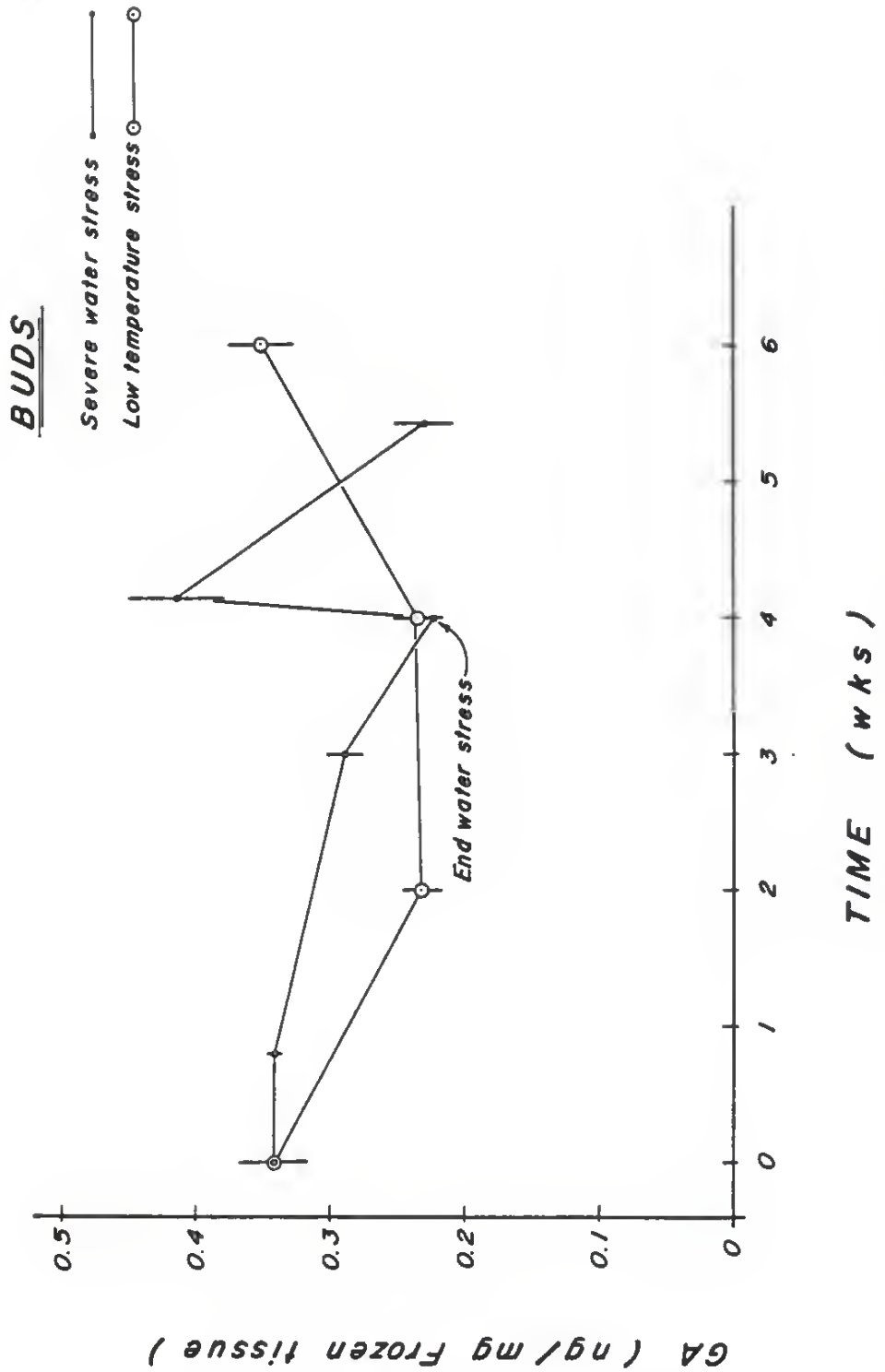

LEAVES
Severe water stress
Low temperature stress
End water stress
nd
GA (ng/mg Frozen tissue)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
TIME (wks)

Figure V.11 Effect of time of water and low temperature stress on GA levels in 'Tahiti' lime buds as measured by the GA_4 radioimmunoassay. The bars indicate standard deviations of triplicate samples. The control value at 0 time represents the control level of GA throughout the measurement time.


BUDS
Severe water stress
Low temperature stress
End water stress
GA (ng/mg Frozen tissue)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
TIME (wks)

value after 4 weeks. This level was similar in magnitude to that observed in leaves after 4 weeks of water stress. One day after water stress was alleviated the GA level rose to a maximum in buds (0.42 ng/mg frozen tissue). The GA level was found to have decreased when measured 10 days after water stress had been alleviated and this level was slightly below that which had been found prior to the beginning of water stress.

Gibberellin levels that were measured in low temperature stressed buds decreased after 2 weeks of stress, but did not differ from those levels measured after 4 weeks. The GA levels measured in buds after either 4 weeks of water or low temperature stress were not different from one another. As the duration of low temperature stress increased to 6 weeks, the GA levels in buds also increased to a maximum of 0.35 ng/mg frozen tissue.

Discussion

The measurement of GAs and the understanding of their role in the flowering process of citrus has been hampered by the lack of sensitive and precise analytical methods which can be used to rapidly process many extracts where limited amounts of tissue are available. The radioimmunoassay reported here allows for detection of as little as 25 pg GA in unpurified extracts.

A problem in developing an immunoassay against GA was producing a GA-BSA conjugate that was capable of eliciting an immunogenic response and an antibody directed against GA. The initial conjugates made by the procedures of Weiler and

Wieczorek (109) and Atzorn and Weiler (3, 4) had low molar amounts of GA_3 coupled BSA. Even by modifying their procedures by adjusting reaction pH or rereacting previously synthesized conjugates, no significant improvement in GA_3 conjugation efficiency to BSA was acheived. Unfortunately, we were not able to produce any GA-directed antibodies when using these conjugates to immunize rabbits. This fact may have resulted from either too few mol GA bound per mol BSA (low molar coupling ratios), or because relatively insensitive techniques were used for initial antisera screening of GA-directed antibodies, or because not enough rabbits were immunized and therefore, the right rabbit had not been selected that would yield a GA-directed antibody in a reasonable amount of time.

The production of GA_3-amino-n-caproic acid-BSA and GA_4-adipic acid-BSA differed from the other GA-BSA conjugates produced because amino-n-caproic acid and adipic acid are 5 and 4 C compounds, respectively, which were used as spacers between GA and BSA. During conjugation of the C 6, COOH of GA_3 to NH_2 of amino-n-caproic, we learned that it was difficult to couple these 2 compounds efficiently. Nevertheless, the amino-n-caproic acid being less bulky than the NH_2-lysine of BSA, and therefore, less sterically hindered, facilitated the coupling process. This observation gave credence to our belief that the C 6, COOH of GA_3 was sterically hindered and this hinderance may have resulted in the low coupling ratios observed as a result of using the mixed anhydride or symmetrical anhydride reactions to couple

GA_3 to BSA. Alternatively, by using adipic acid and coupling it to the only alcoholic group on C 3 of GA_4, the likelihood of steric hinderance reducing the coupling of GA_4 to mono-n-succinimidyl adipate was greatly reduced. The greater amount of GA_4 coupled (59 mol) to BSA lead to the production of GA_4-directed antibodies.

The procedures for characterizing the GA_4 antibody reported here were essentially like those reported previously (3, 4, 113, 114). This GA_4 antibody showed differences from those GA antibodies produced previously. The sensitivity of the GA_4 antibody reported here was 25 pg (range 100 to 1100 pg) versus 2 pg (range 4 to 80 pg) reported by Weiler and Wieczorek (113), but equivalent in sensitivity to that reported by Atzorn and Weiler (3). These results are significant in that no methylation of GA standards was needed in this assay reported here as compared to the required methylation of GA standards in previously reported radioimmunoassays (3, 113). The GA antibodies produced by Atzorn and Weiler (3, 4) were more selective than the GA_4 antibody of these studies as were the GA antibodies produced and reported by Weiler and Wieczorek (31). Apparently, producing a GA_3-BSA conjugate which had coupled the C 6, COOH of GA_3 to NH_2-lysine of BSA resulted in a more selective GA antibody. Nevertheless, for the purposes of measuring GA in citrus, where only GA_1 and GA_9 have been identified, an antibody which was not selective for a specific GA was advantageous to insure that GA levels in

citrus tissues could be quantified, since GA levels in plant tissues are generally low (3, 4, 108, 109, 110, 113).

Little research data has been accumulated with regard to the endogenous levels of GA in citrus tissue. Only GA_1 and GA_9 have been identified and most of the GA measurements made in citrus tissue refer to quantitative levels of GA-like substances. The lack of sensitive, rapid, and accurate assays to measure GA levels in small quantities of citrus tissue have hindered the study of GA control of citrus flowering even though exogenous application of GA has been shown to clearly inhibit flower formation. Another problem in studying citrus flowering is that control over the floral inductive process has been insufficient in that many flowering studies were conducted with trees growing in the field. In studies reported here, we utilized 2 different floral inductive treatments (water and low temperature stress) and measured GA levels in leaves and buds in an effort to correlate GA changes with the floral inductive treatment. The GA level in leaves reached a maximum point 1 week after water stress (Figure V.10). This increase in GA may be a general water stress response and its physiological relevance is presently unknown. However, with the alleviation of water stress, the GA levels measured 1 day later dropped and then recovered to a level above that noted prior to the beginning of water stress. Gibberellin levels measured in leaves during low temperature stress dropped at the 2 week interval to a non-detectable level and then rose to levels obtained at 3 and 4 weeks of water stress and to

levels like those leaves fully recovered from water stress. Apparently, the change in GA levels is subject to the relative change in stress. However, when stress was maintained over a period of time, the GA levels in leaves remained relatively constant. The GA levels reported here are well within the range of those reported previously for equivalents of GA_3 in vegetative shoot tips of citrus (5.0 ng/mg fresh wt) (70), young fruitlets (5.0 ng/mg fresh wt) (23), and flavedo of mature fruit (0.5 ng/mg fresh wt) (23, 29, 32). The physiological relevance of leaf GA as the hormone relates to flowering is unknown. However, it has been shown that leaves are not required for the production of flowers in leafless 'Tahiti' lime cuttings (Chapter III), and therefore, the direct effect of leaves on flowering in citrus may not be significant.

Changes in GA levels in buds was less volatile than that noted in leaves. This observation may have resulted from a more variable response to treatment observed in leaves as compared to buds. The GA levels in buds dropped in a time dependent manner under water stress conditions and then increased when measured 1 day after the alleviation of water stress. The GA level in buds returned to a level less than that measured prior to the beginning of water stress once the tree had fully recovered from water stress and equaled that measured after 4 weeks of water stress. This increase in GA levels in buds noted 1 day after the alleviation of water stress could be involved with promoting the breaking of buds. This may be possible because it had

been noted previously (S.M. Southwick and T.L. Davenport, submitted for publication J. Amer. Soc. Hort. Sci.) that application of high GA concentration (10^{-3} M) to lime trees could promote the breaking of buds, but inhibit their subsequent development. GA levels were dissimilar in buds collected from trees during water stress versus those collected under low temperature stress conditions, except at the 4 week interval. The 4 week interval is critical because it is after 4 weeks of water or low temperature stress when a consistent floral response can be obtained in lime (Chapter III). However, it is unlikely that any critical GA level (high or low) is required for the floral response in lime because flowering can be achieved by periods of water or low temperature stress lasting for as little as 2 weeks (Chapter III). These studies indicate that there is no direct correlation between GA levels and flowering ability. Gibberellin levels in buds do change with time under stress and also vary based upon the type of stress treatment. Therefore, it is likely that the bud itself can control its own destiny with regard to the synthesis and degradation of GA.

We utilized water and low temperature stress to induce flowering in 'Tahiti' lime and found that GA levels in leaves and buds were dissimilar. The GA level measured did not correlate with the flowering habit of lime, nor with the floral inductive stress treatment in ways previously suggested. Although water and low temperature stress treatments regulate flowering in lime, regulation may not occur

by the same molecular mechanism, especially as it relates to endogenous GA level.

The concept of plants having hormones has come under attack in the last 5 to 10 years. Trewavas (97) has been the major attacker and he has suggested that plant hormones be called growth substances because they do not act like animal hormones, but rather, they act like integrating agents in the "plastic" development of plants. Plant growth substances integrate different parts of the growth cycle in a particular organ. Trewavas has also suggested that the measurement and quantification of a plant hormone and then correlation of the hormone level with a developmental response, or change, has led to little progress toward determining the role of plant hormones in growth and development of plants. He has carefully evaluated relevant plant hormone literature and concluded that tissue sensitivity is of greater importance than hormone level in developing an understanding of the molecular action of plant hormones and their effects on plant growth. Tissue sensitivity relates to the strict control of plant hormone receptors, which in turn regulate plant development through altering receptor number, or affinity for the growth hormone. In our studies with GA control of flowering in 'Tahiti' lime, tissue sensitivity was not evident as in other growth and developmental processes which are influenced by plant hormone application. Gibberellin A_3 application inhibited flowering in water stressed, floral-induced and field grown 'Tahiti' lime trees, regardless of

season (17, Southwick and Davenport, unpublished). However, meristematic tissues which eventually become floral shoots are sensitive to GA application.

Trewavas (97) has suggested that gradients of plant hormone levels are commonly found in plant organs as a result of circulation of hormones in the vascular system. Plants have 3 ways of counteracting the effects of this varying concentration. The first is by designing the growth response so that it varies linearly with the logarithm of the plant hormone concentration. The second counteraction is universal biosynthesis of plant hormones, which mitigates the effect of growth substances arriving in the vascular tissue by synthesizing them in the organ itself. Thirdly, plant hormones are chemically designed to pass through membranes, which helps to even out concentration differences over short distances. If these 3 counteractions by plants to varying plant hormone levels are true, then changes of GA and ABA observed in these experiments reported here for 'Tahiti' lime buds, may not be great enough to control water-stress induced dormancy, budbreak, or flowering. Since the tissue sensitivity concept as defined by Trewavas may not relate to floral inhibition in lime by GA, likewise, order of magnitude changes in endogenous hormone levels may not be needed to modify flowering. Possibly, subtle changes in GA concentration or specific GAs could combine with appropriate receptors to regulate flowering. Moreover, it may be a balance between specific GAs and ABA, or other plant hormones that regulate stress induced dormancy, budbreak, and flowering in lime.

CHAPTER VI
SUMMARY AND CONCLUSIONS

Control of flowering in citrus by plant hormones has been investigated only to a limited extent by using field grown trees, applying plant growth hormones, and observing the responses on flowering. Experiments were conducted with container grown 'Tahiti' lime trees in order to characterize conditions needed to induce flowering. Cyclical or continuous water stress for 4 to 5 weeks induced flowering. Moderate (-2.25 MPa, midday) or severe (-3.5 MPa, midday) water stress as measured by leaf xylem pressure potential, for as little as 2 weeks induced flowering, but the response was more significant in severely stressed trees. Low temperature (18^{o}C day/10^{o}C night) stress induced a time (increasing duration of stress) dependent flowering response much like that of moderate water stress. Significantly negative leaf xylem pressure potentials as compared to controls were found only under water stress treatment, suggesting that a common stress-linked event, separate from low plant water potential is involved in floral induction. Leafless, immature cuttings from mature, field-grown trees were induced to flower by water stress treatment, suggesting that leaves are not essential for a flower inductive response.

A radioimmunoassay (RIA) was developed for the measurement of (±) abscisic acid (ABA). The assay sensitivity ranged from 25 to 1000 pg (±) ABA/assay and was used to

measure total ABA in unpurified extracts of leaves and buds collected from 'Tahiti' lime trees which had been grown under the floral inductive conditions of severe water or low temperature stress. The levels of free ABA were measured in these same tissues by separating free ABA from ABA-conjugates by thin layer chromatography prior to quantitation by the ABA RIA. The levels of total ABA measured by RIA in leaves indicated that ABA was higher in leaves during water stress than during low temperature stress treatment. Abscisic acid levels decreased when water stress was alleviated by rewatering. The levels of free ABA showed similar patterns of change when compared to total ABA levels of water and low temperature stressed leaves. The levels of free ABA, however, were 4- to 10-fold less than total ABA and indicated that ABA conjugates were found at high levels in leaves of 'Tahiti' lime. The importance of ABA measurements in leaves is questionable since experimental results obtained from earlier studies indicated that flowering could be induced by water stress in leafless, non-induced cuttings of 'Tahiti' lime.

The levels of total ABA measured in buds of 'Tahiti' lime were quantitatively similar to those measured in leaves under the conditions of water and low temperature stress. The total ABA levels in buds dropped after alleviation of water stress, but not to the same extent as they did in leaves. The level of free ABA in buds dropped to lower levels than those measured prior to the beginning of stress and fell sharply after alleviation of water stress. Free

ABA levels were lower in buds of trees grown under low temperature conditions. Free ABA levels were 15- to 35-fold less than total ABA levels in buds indicating that a high level of ABA-conjugates were produced in buds. The increase in ABA levels during water stress and their decline as buds were released from water stress correlated reasonably well with ABA's physiological role in dormancy and budbreak in other tree species. The levels of ABA were dissimilar when measured in leaves or buds as a result of water or low temperature stress. Although the 2 stress treatments regulate flowering in 'Tahiti' lime, they do not appear to do so through similar regulation of ABA levels.

Abscisic acid glucose ester (ABA-GE) was synthesized for use in cross reactivity studies with the ABA antisera by reacting the free acid of ABA with $CsHCO_3$ to form the Cs salt of ABA. The Cs salt of ABA was then combined with acetobromo-a-D-glucose tetraacetate, and the tetraacetate derivative of abscisic acid glucose ester was formed. Acetate groups were selectively removed from the glucose moiety with a crude enzyme preparation derived from Helianthus annuus seeds. Abscisic acid glucose ester was purified via silica gel column chromatography and identified by NMR. The preparation of plant hormone esters by use of $CsHCO_3$ is simple and the reaction occurs under mild conditions. Purification of ABA-GE by silica gel column chromatography is rapid and easily scaled up. The preparation of other plant growth hormone esters should be achievable by utilizing this procedure.

Gibberellin (GA) immunogens were synthesized by several different procedures. An immunogen produced by conjugating gibberellin A_4 (GA_4) to adipic acid and then coupling this conjugate to bovine serum albumin yielded antisera suitable for use in a GA RIA. The GA_4 RIA was sensitive in the range of 100 to 1100 pg GA_4/assay. The antisera cross reacted significantly with GA_1, GA_7, and GA_9 and the antisera was therefore used to measure GAs in 'Tahiti' lime leaves and buds collected from trees growing under the floral inductive conditions of water and low temperature stress. The levels of GA measured in water stressed leaves did not coincide with those GA levels measured in low temperature stressed leaves. The levels of GA measured in buds collected from trees growing under water stress or low temperature stress conditions were also dissimilar. The GA levels measured in buds were slightly higher than those levels measured in leaves. Gibberellin levels decreased in leaves and increased in buds when water stress was alleviated by rewatering trees. The increase in GA levels in buds corresponded to the breaking of water stressed-induced dormancy, and higher GA levels may play a role in budbreak. Although water and low temperature stress treatments can induce flowering in lime, there is not a common change in GA levels due to treatment. This fact would indicate that although GA levels may play a role in controlling flowering in 'Tahiti' lime, the mechanism of floral control by water or low tempeature stress does not function through similar changes in GA levels. This role for GA in budbreak may be

possible because other experiments (S.M. Southwick and T.L. Davenport, submitted for publication, J. Amer. Soc. Hort. Sci.) indicated that GA application at 10^{-3}M concentration promoted budbreak, but not shoot elongation in 'Tahiti' lime. A better correlative role for ABA than GA for control of flowering, water stress-induced dormancy and budbreak of 'Tahiti' lime was found in these studies. No direct correlation with ABA or GA levels in lime buds or leaves could be made for low temperature-induced flowering.

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Mr. Southwick was born in Woodland, California. He was raised in Amherst, Massachusetts, and attended Amherst Regional High School. After graduation he enrolled at Cornell University in Ithaca, New York, and graduated with a Bachelor of Science degree from the College of Agriculture and Life Sciences. Mr. Southwick enrolled at the University of Florida upon graduation from Cornell and received a Master of Science degree in horticultural science with a concentration in plant physiology from the Department of Fruit Crops. He entered the Ph.D. program in plant physiology/biochemistry at Washington State University in Pullman, Washington, in the fall of 1980 and finished his coursework toward the Ph.D. degree. Mr. Southwick left Washington State University in 1982 to pursue several business entrepreneurial endeavors in Florida and returned to finish his Ph.D. research at the University of Florida in January, 1984.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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